The Hydrophobin EAS Is Largely Unstructured in Solution and Functions by Forming Amyloid-Like Structures

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Summary

Background: Fungal hydrophobin proteins have the remarkable ability to self-assemble into polymeric, amphipathic monolayers on the surface of aerial structures such as spores and fruiting bodies. These monolayers are extremely resistant to degradation and as such offer the possibility of a range of biotechnological applications involving the reversal of surface polarity. The molecular details underlying the formation of these monolayers, however, have been elusive. We have studied EAS, the hydrophobin from the ascomycete Neurospora crassa, in an effort to understand the structural aspects of hydrophobin polymerization.

Results: We have purified both wild-type and uniformly ¹⁵N-labeled EAS from N. crassa conidia, and used a range of physical methods including multidimensional NMR spectroscopy to provide the first high resolution structural information on a member of the hydrophobin family. We have found that EAS is monomeric but mostly unstructured in solution, except for a small region of antiparallel β sheet that is probably stabilized by four intramolecular disulfide bonds. Polymerised EAS appears to contain substantially higher amounts of β sheet structure, and shares many properties with amyloid fibers, including a characteristic gold-green birefringence under polarized light in the presence of the dye Congo Red.

Conclusions: EAS joins an increasing number of proteins that undergo a disorder—order transition in carrying out their normal function. This report is one of the few examples where an amyloid-like state represents the wild-type functional form. Thus the mechanism of amyloid formation, now thought to be a general property of polypeptide chains, has actually been applied in nature to form these remarkable structures.

Introduction

Hydrophobins are a family of low molecular weight (7–9 kDa) cysteine-rich proteins that have unusual biophysical properties. They are highly surfactant molecules that can polymerize at an air/water or water/oil interface, forming a robust, polymeric monolayer that is able to reverse the hydrophobicity of surfaces, both in vivo and in vitro [1]. These proteins are produced and secreted by filamentous fungi and are distinguished by the conserved pattern of cysteines in the consensus sequence [2]:

\[
X_{0.38} - C - X_{0.67} - C - X_{11.44} - C - X_{0.27} - C - X_{6.15} - C - X_{0.82} - C - X_{0.14}
\]

The genes coding for over forty hydrophobins have been cloned and the expressed products have been shown to play several key roles in fungal development [1, 3–7]. Most significantly, they act to allow the emergence of aerial hyphae from the mycelial growth medium and to provide a water-repellent coating to spores that are aerily dispersed. These functions are brought about through polymerization of the secreted hydrophobins at the air-liquid interface into rodlets (fibrils with dimensions of around 10 × 200 nm), which aggregate into an amphipathic monolayer on the hyphal or spore surface [8, 9]. The inner face of this monolayer is hydrophilic in nature, while the outer face is highly hydrophobic; it is this hydrophobicity that confers water-repellency on the hypha/spore.

The rodlets are exceptionally robust, and hydrophobins have been divided into class I and class II types on the basis of their hydrophobicity plots and the solubility characteristics of the rodlets that they form [2]. Class I proteins are insoluble even under conditions such as boiling 1% SDS, and can be solubilized only by treatment with solvents such as 100% trifluoroacetic acid (TFA) [10–12], while class II rodlets can be dissociated under milder conditions [13]. Despite the apparent harshness of these treatments, it has been demonstrated that SC3, a hydrophobin from the mushroom Schizophyllum commune, is capable of reforming rodlets in vitro after TFA extraction; indeed, the process of dissociation and reformation of the rodlets can be carried out multiple times [11]. The reconstituted hydrophobins appear to have the same microscopic appearance and physical properties as native rodlets [10, 11].

The ability of hydrophobins to assemble into robust monolayers (around 10 nm thick) and alter the hydrophobicity of a surface has lead to suggestions that these proteins may have a range of potential uses as biotechnological tools [2]. It has already been demonstrated that rodlets may be formed in vitro on both hydrophobic (e.g., Teflon) and hydrophilic (e.g., nitrocellulose) sub-
strates, with the like face of the rodlets adhering to the surface and the other face directed outwards [14]. Thus, for example, hydrophobic surfaces may be coated with rodlets to allow the subsequent attachment of cells or proteins in biosensor design, or to increase their biocompatibility in applications such as tissue engineering. The latter use would be assisted by the observation that humans have been ingesting hydrophobins from mushrooms and other edible fungi for many years without apparent side effects [2].

SC3 is currently the best-characterized member of the hydrophobin family. Circular dichroism and infrared spectroscopic studies revealed that the protein appears to form some β sheet structure in solution and that the amount of β sheet is increased upon polymerization [15]. In addition, the formation of an α-helical domain was detected upon binding SC3 to a solid hydrophobic support. Preliminary characterization of a class II hydrophobin, cerato-ulmin, has been reported [16]. Yaguchi and co-workers proposed a tentative disulphide bond arrangement for this protein and alluded to unpublished NMR data that suggested the presence of both some antiparallel β sheet in the cysteine-containing regions of the polypeptide, and a short stretch of α-helix.

Aside from these studies, very little is known regarding the molecular details of hydrophobin structure and polymerization. This is due in part to the difficulties in extracting and handling these highly surfactive proteins. We have sought to address this deficit by examining the structure of EAS, the hydrophobin from Neurospora crassa [17, 18], using a range of biophysical methods. Cloning and sequence analysis of the eas gene (named for the easily wettable phenotype of the EAS-deficient mutant) revealed that the predicted protein product is a class I hydrophobin with the characteristic distribution of hydrophobic and conserved cysteine residues [19, 20].

Here, we demonstrate the existence of several isoforms of EAS in purified rodlets and show that each isoform is capable of forming native-like rodlets in the absence of the others. Sedimentation equilibrium experiments reveal that two of the isoforms are monomeric in solution, while data from near-UV circular dichroism measurements suggest that, under a range of solution conditions, there is very little regular secondary structure present in any of the isoforms. Interestingly, analysis of both homo- and heteronuclear NMR data reveals that, in solution, EAS displays a small core of antiparallel β sheet, probably stabilized by the four disulfide bonds. The remainder of the protein appears to be largely unstructured, judging from chemical shift, coupling constant and NOE data, together with (1H)15N NOE measurements. We further show that treatment of EAS rodlets with the dye Congo Red results in the appearance of gold-green birefringence, a property associated with amyloid fibers. Consistent with this, solid-state CD spectra suggest that EAS rodlets contain a substantial amount of β sheet structure. This is one of only a few naturally occurring examples of amyloid-like structure that is not related to a disease state, and may constitute a useful model system from which to study the properties of amyloid formation and structure.

Figure 1. Purification and Amino Acid Sequence of EAS
(a) Plot of absorbance at 218 nm against elution time for the reversed-phase HPLC purification of disassembled N. crassa rodlets. Peaks corresponding to isoforms Ia, Ib, and II are labeled.

(b) Amino acid sequence of EAS. The leader sequence is shown in italics, a black triangle indicates the alternate cleavage site for the mutant) revealed that the predicted protein product is a class I hydrophobin with the characteristic distribution of hydrophobic and conserved cysteine residues [19, 20].

Results

Purification and Identification of EAS Isoforms
EAS was purified from N. crassa cultures as described previously [12]. The key step in this procedure is disassembly of the intact rodlets with neat TFA at 0 °C [21]. Following this step, reversed phase HPLC revealed three major peaks [12], which we have termed fractions Ia, Ib and II and which could be separated using shallow solvent gradients (Figure 1a). Electrospray mass spectrometry revealed that fraction Ia has a molecular mass of 8180 (±1) Da, consistent with the mass for the predicted protein product of the eas gene if it is post-translationally cleaved at the Arg(-1)-Ala1 peptide bond (Figure 1b). This was confirmed by N-terminal sequence analysis. The mass of fraction Ib is 8196 (±1) Da (i.e., equal to the mass of Ia + 16 Da), and we have so far been unable to determine the exact nature of the 16-Da addition. Efforts to identify this modification are continuing. Mass spectrometric analysis of fraction II revealed that it actually comprised two species of similar abundance, with molecular masses of 7908 Da and 7925 Da. The first of these corresponds well to the expected mass for EAS with an alternative signal peptide cleavage site between Thr3 and Ile4 (Figure 1b). It is likely that the second
species contains a 16-Da modification, as observed for fraction Ib. It is interesting to note that the predicted leader sequence cleavage site is between Ala(-9) and Ala(-8) [22]. This suggests that EAS may be secreted as a pro form that is subsequently cleaved by a second protease. Reaction of purified EAS with 1 M iodoacetamide, followed by acid hydrolysis and amino acid analysis did not reveal the presence of carboxymethylcysteine. It is therefore likely that the eight cysteine residues are involved in intramolecular disulfide bonds.

All EAS Isoforms Form Rodlets

Having obtained purified EAS Ia, Ib and II, we first sought to determine whether these purified fractions were capable of forming rodlet structures in the absence of the other fractions. Samples of each isoform were spotted onto a clean mica surface and the water allowed to evaporate. Figure 2 shows atomic force micrographs of the structures formed in each case; it can be seen that all fractions form rodlets. The form of these rodlets is also very similar to that of the wild-type spore surface [17, 23]. These data demonstrate that the protocol used to purify EAS yields functional protein, and that neither the alternate cleavage sites nor the 16-Da mass addition are essential for, or detrimental to, the formation of rodlets. The function of these modifications remains unclear at present.

Initial Characterization of EAS Isoforms

We next used circular dichroism (CD) spectropolarimetry to assess the secondary structure content of each isoform. Samples of the EAS isoforms could be prepared in a variety of solvent conditions and were generally stable at room temperature for several months. The samples were, however, very sensitive to agitation and any other process that created substantial air-solvent interfaces, and would rapidly form rodlets structures at the exposed interface under these circumstances. Figure 3 shows CD spectra of EAS Ia and II under a range of solvent conditions, including water alone, 30% acetonitrile containing 0.1% TFA (pH 2), and phosphate (pH 6.2) buffers. The spectra are similar under all conditions and are consistent with EAS Ia containing little or no regular secondary structure. Spectra of EAS Ib recorded under corresponding conditions gave similar results (data not shown).

In an attempt to probe the secondary structure of the rodlet form of EAS, samples of Ia and Ib were each applied to the face of quartz cuvettes and the solvent allowed to evaporate. A CD spectrum of the dried-down EAS Ia sample is shown in Figure 3. Its shape is comparable to spectra observed previously for oriented \( \beta \) sheet films [24], suggesting the presence of substantial amounts of \( \beta \) sheet structure in the EAS rodlets. The spectrum of dried-down EAS II (Figure 3b) had an identical shape, indicating that it also formed a \( \beta \) sheet film.

Given that the rodlet and solubilized forms of EAS give such different CD spectra, we next examined the aggregation state of EAS in solution. Sedimentation equilibrium experiments revealed that both EAS Ia (Figure 4) and EAS Ib (data not shown) are monomeric in solution over the range of concentrations used. EAS II, however, sedimented to the bottom of the centrifuge cells, indicating that it was forming large aggregates. We were unable to determine whether these aggregates were rodlets, although we suspect that they are not,
Structure

Figure 4. Sedimentation Equilibrium Data for EAS Ia, Demonstrating that the Protein is Monomeric in Solution

A plot of weight-average molecular weight against protein concentration is shown, generated from data recorded at 56,000 rpm (22°C, loading concentration 220 μM).

given that hydrophobins generally form rodlets at air-water interfaces.

**NMR Spectroscopy Reveals Small Amounts of β Structure in EAS Ib**

In order to obtain a picture of the conformational preferences of EAS isoforms in solution at much higher resolution, we prepared samples of both unlabeled and uniformly 15N-labeled EAS fractions Ia, Ib, and II for NMR experiments. One-dimensional (1D) 1H NMR and two-dimensional 15N-HSQC spectra of EAS II displayed very broad signals, consistent with the sedimentation equilibrium results, which indicated that it was forming soluble aggregates. No further experiments were carried out on this isoform. In contrast, the 1D 1H NMR spectra of EAS Ia and EAS Ib both displayed relatively narrow line widths and a reasonable degree of chemical shift dispersion (Figure 5a). The spectra of these two isoforms exhibit similar features, suggesting that they exist in related conformational states in solution. 15N-HSQC spectra of Ia and Ib also display many similarities, although the quality of the spectrum from Ib (Figure 5b) was higher overall, and subsequent structural studies focused on this isoform. It is notable that NMR samples of all EAS isoforms showed very fine needle-like fibers, presumably rodlets, at the sample meniscus, particularly in situations where the sample was perturbed (for example, by adding buffer components or adjusting the pH).

Using a combination of homonuclear 2D and 15N-separated 3D experiments, we were able to obtain assignments for ~91% of the backbone proton and nitrogen nuclei, and 80% of the sidechain protons. Further assignments of aliphatic resonances were prevented by spectral overlap, caused by the large number of methyl-containing residues (6 Leu, 6 Ile, 7 Ala, 7 Val, 5Thr). Figure 6a shows a plot of the chemical shift index [25] for H\(^+\) protons in EAS Ib; this binary parameter provides an indication of which regions of the protein chain exist in predominantly α-helical or β-sheet conformations. A sequence of three or more residues with values of +1 (intervening values of 0 are allowed as long as they do not constitute more than 25% of the sequence) is indicative of a β strand, while a sequence of four or more values of −1 suggests the presence of an α-helix. While no regions that fulfill the requirements for α-helix are present in EAS Ib, three β-strand regions are evident (marked with arrows). It is notable that two of these coincide with sequences that contain at least one cysteine residue (marked with circles).

The value of the H\(^n\)-H\(^n\) scalar-coupling constant (\(\mathcal{J}_{\text{HNH}}\)) provides another measure of the conformational preferences of individual residues. Values of greater than 8 Hz are correlated with β-type structure, while values less than 6 Hz indicate a helical conformation. Intermediate values are generally associated with conformational averaging (as would be seen in an unstructured polypeptide). We have converted the coupling constants measured from an HNHA spectrum of EAS Ib into a binary format, where \(\mathcal{J}_{\text{HNH}} > 8\) Hz is represented as +1, \(\mathcal{J}_{\text{HNH}} < 6\) Hz is represented as −1 and 6 < \(\mathcal{J}_{\text{HNH}} < 8\) Hz is represented as 0; this ‘coupling constant index’ is plotted in Figure 6b against residue number. It can be seen that, while many residues appear to have intermediate coupling constants, a number of residues have values that are consistent with a β conformation, and...
Figure 6. Indicators of the Secondary Structure Composition of EAS Ib

Arrows indicate identified \( \beta \)-strands, while cysteine residues are identified both by blue dots and by cyan bars.
(a) Plot of \( H^+ \) chemical shift index versus residue number. Values of \( +1 \) indicate \( \beta \) sheet, while values of \( -1 \) indicate the presence of \( \alpha \)-helical secondary structure.
(b) Graph of “coupling constant index” against residue number. The significance of \( +1 \) and \( -1 \) values is the same as in (a).
(c) Plot of \( \langle H^+ \rangle \) NOE versus residue number. Smaller values indicate increased local motion at that backbone amide unit.

that the highest concentrations of such residues again occur around cysteine residues.

In order to further probe the conformational preferences of EAS Ib, we measured the \( \langle H^+ \rangle \) heteronuclear NOE for each residue. This parameter provides an indication of the degree of fast local motion taking place at each backbone H-N bond vector (that is, motion that is substantially faster than the overall tumbling of the protein). Values of the NOE that approach 1 signify H-N groups that are rigidly held within the protein structure, while smaller values, down to \(-3.6\) (in the absence of chemical exchange processes) indicate increasing degrees of high frequency (picosecond timescale) local motion. The plot of \( \langle H^+ \rangle \) NOE against residue number shown in Figure 6c differs markedly from the types of plots typically observed for well-structured proteins. Segments of EAS Ib (e.g., residues 22–43 and 61–75) appear to be undergoing significant degrees of local motion, and only a few smaller regions appear to be relatively rigid. The less mobile regions again correspond to the cysteine-containing sequences in the protein. These data strongly support the conclusion that EAS Ib is largely flexible in solution, with the exception of a small disulfide-bonded core.

Definition of the \( \beta \) Sheet Structure in EAS Ib

A detailed analysis of 2D NOESY and 3D \( ^{15} \)N-NOESY-HSQC experiments recorded on EAS Ib yields a picture of EAS structure that is consistent with that proposed above. Thus, only small pockets of the sequence give rise to the majority of the medium or long-range connectivities associated with a folded structure; many regions display only intraresidue and sequential NOEs. In total, 520 non-redundant NOEs (intra- and inter-residue) could be assigned from the 2D and 3D data, and this number proved insufficient to obtain good convergence from structure calculations using the DYANA [26] or CNS [27] software, even in combination with coupling constant information obtained from the HNHA experiment. Despite this, a representation of the \( \beta \) sheet secondary structure can be built up from an analysis of the assigned NOEs (Figure 7). The core of this structure comprises a three-stranded all-antiparallel sheet, involving residues Tyr14–Cys19, Ser56–Lys62 and Cys80–Ala82. The interstrand \( d_{\text{inter}} \) NOEs Cys18–Val58, Pro16–Cys60 and Cys61–Cys80 were important in defining this structure, together with a number of interstrand \( d_{\text{intra}} \) NOEs. In addition, a number of connectivities can be observed between Tyr17 and the residues surrounding Cys45, and also between these latter residues and amino acids in the Ser56–Lys62 and Cys80–Ala82 strands of the core.
Figure 8. EAS Forms Structures with Amyloid Fibril Characteristics
EAS rodlets were allowed to assemble on silica glass plates and stained with an aqueous 0.5% (w/v) solution of Congo Red.
(a) Reconstituted EAS Ia rodlets.
(b) Reconstituted EAS II rodlets.
(c) Native rodlets. Images are shown as viewed through cross polarizers on a light microscope.

sheet. Consideration of the amino acid sequence of EAS reveals that essentially all of the long range NOEs involve pairs of residues that are near cysteine in the primary structure. These data are therefore in accord with the iodoacetamide protection data, suggesting that the cysteine residues are indeed involved in disulfide bond formation.

These conclusions fit with all of the chemical shift, coupling constant and \(^{1}H\)\(^{15}N\) NOE data and present a picture of EAS solution behavior in which four disulfide bonds hold together a core of \(\beta\) sheet structure with large unstructured loops connecting the elements of the core.

Does EAS Form Functional Amyloid Fibers?
Given that, in solution, EAS appears to contain a core of \(\beta\) sheet structure, and that it is able to form polymeric structures, we sought to investigate the possibility that EAS forms amyloid-like structures. Rodlets were allowed to assemble from aqueous solutions of either EAS Ia or II onto a silica glass surface and were then stained with Congo Red dye. From visual inspection, it was clear that Congo Red bound to the rodlets and, under polarizing light, it could be seen (Figure 8a,b) that the EAS rodlets do indeed exhibit the gold-green birefringence characteristic of amyloid fibrils [28]. In addition, native rodlets treated in the same way also displayed comparable birefringence (Figure 8c). This, together with the dimensions of the fibrils (~10 nm in diameter, compared to typical values of 5–13 nm for other amyloid structures [29]) and the high \(\beta\) sheet content observed in CD spectra of deposited EAS rodlets (Figure 3), strongly suggests that EAS rodlets (both native and reconstituted) bear a close resemblance to amyloid fibers, such as those observed in a wide range of mammalian disease conditions. We also note that, during the preparation of this manuscript, a similar observation was reported as unpublished results during a study on the involvement of the disulfide bonds on SC3 rodlet formation [30].

Relating Structure to Function
One of the primary functions of hydrophobins is to provide a hydrophobic coating to fungal spores and fruiting bodies, and a long-standing question exists as to how they are able to carry out this function. The present study provides the first atomic resolution information regarding the relationship between structure and function in this remarkable family of proteins. The mature EAS protein, following cleavage of a signal peptide, exists in solution as a largely flexible monomer, with the exception of a core of antiparallel \(\beta\) sheet that is most probably stabilized by four intramolecular disulfide bonds. This mostly unstructured protein is able to rapidly self-associate under appropriate conditions (generally the presence of an air-solvent interface) to form highly ordered structures. EAS (and most likely other hydrophobins) shares this property of a functional disorder-order transition with an increasing number of proteins, of which one well-characterized example is the SNARE complex involved in neurotransmission [31]; synaptobrevin is almost totally unstructured in isolation, but folds to form a heterotetrameric coiled-coil in the presence of its binding partners syntaxin and SNAP25 [32].

EAS rodlets display many of the characteristic properties of amyloid fibers. These fibers, first described over 100 years ago, are associated with a substantial number of human disease conditions, including Alzheimer’s, Parkinson’s and Huntington’s diseases [33]. Despite the fact that these fibers have thus far eluded atomic resolution structural analysis, X-ray diffraction [34] and cryo-electron microscopy [35] studies of fibers have revealed a cross \(\beta\) structure with an overall helical twist; all other fibers appear to share this overall structural arrangement [36]. Amyloid-like fibers are usually only observed in abnormal proteins or under destabilizing conditions (e.g., low pH, elevated temperatures, or the presence of chemical denaturants), although it appears that these structures may have functional roles in certain cases. For example, the [URE3] genetic element of Saccharomyces cerevisiae appears to be an amyloid-forming prion protein [37], and it was recently reported that chorian, the major component of silkmoth eggshell, forms an amyloid structure in vivo [38].

The Congo-Red staining and CD spectra also suggest that \(\beta\) sheet is the predominant element of secondary structure in polymerized hydrophobin rodlets. In contrast to amyloid fibrils, however, there is no evidence that rodlets have an overall helical structure. It is difficult to envisage how a helical twist could give rise to an amphipathic membrane. Further, the rodlets appear to
A picture of the mechanism of amyloid formation is gradually being built up (see, for example, [29]). It is suggested that all amyloid formation must begin with a partially or totally unfolded precursor, in order that the backbone amide moieties are available for intermolecular hydrogen bonding across β strands. Current theories suggest that monomeric amyloidogenic intermediates first self-assemble into relatively small aggregates (pro-totifibrils or structural nuclei) with an unfavorable equilibrium constant [39]. This step results in a lag phase in the kinetics of fiber formation. Once these nuclei are formed, growth of the fibers is relatively rapid through the stepwise addition of more monomers [29]. The formation of EAS rodlets, on the other hand, appears to be almost instantaneous (in vitro, at least). It is possible that EAS avoids this lag phase by having the four disulfide bonds to direct formation of the β sheet core observed in our study and thereby guide polymerization. Thus, by orienting the free β sheet edges at a hydrophobic interface, the core may provide a framework that imposes limits on the dimension perpendicular to the direction of rodlet growth.

An additional question concerns the mechanism (if any) through which the fungus prevents intracellular rodlet formation, which would obviously be detrimental to the organism. One possibility is that the disulfide bonds are not formed until the protein is exported to the cell wall, and therefore the structural core may be absent, preventing polymerization. Consistent with this, it is well known that the space between the cell wall and cell membrane of lower organisms contains a number of proteins with disulfide bond isomerase activity (for example, the DsbA protein in Escherichia coli [40]). However, a recent study demonstrated that, at least under some conditions, the SC3 hydrophobin is capable of forming rodlets with all cysteine residues reduced and iodoaceticamide-protected [30]. It is not clear whether this modification affects the kinetics of rodlet formation. Alternatively, cleavage of the leader sequence may be a prerequisite for rodlet formation. This situation is reflected in the formation of amyloid plaques in Alzheimer’s disease; the constituent of these fibers is a 42-residue peptide from the Alzheimer’s precursor protein (APP), that is amyloidogenic only after proteolytic cleavage of the remainder of the protein [41]. Finally, the role of the air-water interface in hydrophobin polymerization remains undetermined, although it is evidently important, acting as a catalyst in the formation of rodlets. It is conceivable that preferential alignment of the monomeric EAS molecules at the meniscus acts as a catalyst for rodlet formation. It is clear that further studies are needed to elucidate these details of hydrophobin function.

Biological Implications

A picture of EAS structure and functional mechanism has emerged from this study. It appears that this extraordinary protein undergoes a disorder—order transition to form amyloid-like structures on the conoidal surface of N. crassa that resemble amyloid fibers and act as an extremely effective water repellent coating. The formation of these polymeric fibers is most likely directed by a small core of disulfide-bond stabilized antiparallel β sheet, and possibly also by the timely cleavage of the pro-sequence. It is notable that the presence of an interface appears to be required for rodlet formation, and in this way rodlets may be distinguished from other amyloid fibers. The robust and reversible assembly of hydrophobin rodlets may well serve as a useful model system in which to further examine the details of amyloid fiber formation and structure.

It is now thought that the ability to form amyloid-like structures constitutes a general property of polypeptide chains, and there are well over 50 examples of proteins and peptides for which amyloid formation can be induced [33]. However, the formation of these structures is normally associated with a disease state (induced, for example, by mutation or by the influence of prion-type agents [42]). In the case of fungal hydrophobin proteins, nature appears to have harnessed this apparently general mechanism for a useful purpose.

Experimental Procedures

Growth of N. Crassa and Purification of EAS

N. crassa STA 4 was grown on solid Vogel’s media supplemented with 2% sucrose in 2 l beakers as described previously [12]. For the preparation of 15N-labeled EAS, cultures were grown on Vogel’s -N media with 2% glucose and 0.1% (15NH4)2SO4 (Cambridge Isotope Laboratories, Andover, MA). Rodlets, solubilized by incubation in 100% TFA, were resolved by reverse-phase HPLC using a Jupiter preparative C4 column (Phenomenex, Torrence, CA) with a 35%–65% gradient of acetonitrile:water containing 10% methanol and 0.1% TFA. Positive fractions were lyophilized and stored at 4°C.

Atomic Force Microscopy

A Nanoscope IIIa multimode scanning probe microscope (Digital Instruments, Santa Barbara, CA) equipped with an AS-130 “J” scanner (125 μm² lateral range, 5.0 μm vertical range) was used for all experiments. Either TESP (Tappingmode Etched Silicon Probes. Digital Instruments) or SC11 noncontact mode tips (Silicon-MDT, Moscow, Russia) with a spring constant of approximately k ~ 48 N/m were used.

Stock solutions (1 mg/ml) of freeze-dried HPLC purified monomer EAS isoforms were freshly prepared, dissolved in Milli-Q water and diluted to 10 μg/ml. A 20 μl aliquot of the resulting solution was deposited on freshly cleaved mica and the water was allowed to naturally evaporate overnight in covered containers. The rodlet film adsorbed to the mica sufficiently well to allow AFM imaging to be carried out in air.

Mass Spectrometry

Electrospray mass spectra were recorded on a Finnegan MAT TSQ mass spectrometer fitted with an ESI interface and operating in positive ion mode with a probe voltage of 4.5 kV. The heated capillary was at 20 V and 250°C, and a sheath gas of ultrahigh purity nitrogen (at 55 psi) was employed. EAS isoforms were dissolved in methanol:water:acetic acid mixture (50:50:1) and infused directly into the spectrometer using a Harvard Apparatus Syringe Infusion Pump 22. Spectra were collected in full scan mode over an m/z 4000 mass range.

Circular Dichroism Spectropolarimetry

CD spectra were recorded on a Jasco J-720 spectropolarimeter using a 1 mm quartz cuvette. In each case, spectra comprised the
sum of three successive spectra with a step size of 0.5 nm, a 1 s response time and a 1 nm bandwidth. Data were acquired at 20°C and were baseline-corrected by subtraction of a spectrum of the buffer. For solution phase spectra, EAS isoforms were dissolved in Milli-Q water and then diluted into one of the following buffers (final buffer concentrations indicated): (i) water alone, (ii) 30% acetonitrile containing 0.1% TFA, (iii) 20 mM sodium dihydrogen phosphate (pH 6.2), (iv) 20 mM sodium dihydrogen phosphate (pH 6.2) containing 150 mM NaCl. For the solid-phase spectrum, a sample of each EAS isoform dissolved in water was laid on the exterior surface of a quartz cuvette and the water allowed to evaporate. Once dried, a CD spectrum was recorded using the parameters above.

**Sedimentation Equilibrium**

Sedimentation equilibrium experiments were carried out on a Beckman XL-A analytical ultracentrifuge. EAS isoforms were dissolved in water (pH 4.5) to give loading concentrations between 20 and 220 μM. Data were recorded at 298 K using six-channel centerpieces and an An-60ti rotor spinning at 56,000 rpm. Data were acquired as absorbance versus radius scans (at 280 and 360 nm) at 0.001 cm intervals and as the sum of ten scans. Scans were collected at 3 hr intervals and compared to determine when the samples had reached sedimentation and chemical equilibrium. After subtraction of the 360 nm scans, the datasets from all speeds and loading concentrations recorded at 280 nm were fitted simultaneously to single-species and homoassociation models in NONLIN [43]. The same software was used to generate weight-average molecular weight plots.

**NMR Spectroscopy**

Samples of EAS isoforms la and lb (both natural abundance and 15N-labeled) were prepared in the same solvents used for CD studies, with the addition of 5% D,O and 20 μM trimethylsilylpropionic-2,2,3,3-d4 acid. Protein concentrations ranged between 100 and 500 μM in 500 μl of solvent. Samples of both unlabeled and 15N-labeled fraction II were likewise prepared, but only in water. All NMR spectra were recorded at either 280 or 298 K on a Bruker DRX600 NMR spectrometer equipped with a triple resonance (HCN) probehead and three-axis pulsed field gradients. Samples of EAS that were maintained at higher temperatures than this were prone to precipitation over periods of hours or days. Initially, one-dimensional 1H and two-dimensional 1H-15N heteronuclear NOE and three-dimensional 15N-TOCSY-HSQC spectra were recorded on all three 7. Stringer, M.A., Dean, R.A., Sewall, T.C., and Timberlake, W.E. fraction II were likewise prepared, but only in water. All NMR spectra were used for all experiments, as described previously. 11. Woeste, H.A.B., de Vries, O.M.H., and Wessels, J.G.H. (1993). Hydrophobins, from molecular structure to multiple functions in fungal development. Mycosenosis 38, 363–374.

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