Recruitment of Class I Hydrophobins to the Air:Water Interface Initiates a Multi-step Process of Functional Amyloid Formation*□

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Vanessa K. Morris†1, Qin Ren†, Ingrid Macindoe‡1, Ann H. Kwan§, Nolene Byrne‡, and Margaret Sunde†3

From the †School of Molecular Bioscience, University of Sydney, New South Wales 2006 and the ‡Centre for Material and Fibre Innovation, Geelong Technology Precinct, Deakin University, Victoria 3217, Australia

Class I fungal hydrophobins form amphipathic monolayers composed of amyloid rodlets. This is a remarkable case of functional amyloid formation in that a hydrophobic:hydrophilic interface is required to trigger the self-assembly of the proteins. The mechanism of rodlet formation and the role of the interface in this process have not been well understood. Here, we have studied the effect of a range of additives, including ionic liquids, alcohols, and detergents, on rodlet formation by two class I hydrophobins, EAS and DewA. Although the conformation of the hydrophobins in these different solutions is not altered, we observe that the rate of rodlet formation is slowed as the surface tension of the solution is decreased, regardless of the nature of the additive. These results suggest that interface properties are of critical importance for the recruitment, alignment, and structural rearrangement of the amphipathic hydrophobin monomers. This work gives insight into the forces that drive macromolecular assembly of this unique family of proteins and allows us to propose a three-stage model for the interface-driven formation of rodlets.

Class I hydrophobins are a family of small amphipathic proteins that are produced by filamentous fungi in a monomeric form but are able to self-assemble into amphipathic monolayers composed of amyloid-like structures known as rodlets (1–3). The polymerization of the hydrophobins occurs on contact with a hydrophobic:hydrophilic interface, such as an air:water boundary or when hydrophobins are secreted from the spores and come into contact with the air. Members of the hydrophobin family are characterized by the presence of four disulfide bonds. The amphipathic nature of hydrophobins drives them to the surface of solutions, where they reduce the surface tension (1).

Some of the functional roles of the class I hydrophobins include acting as a surfactant at the air:water boundary to reduce the surface tension, which is a barrier to aerial growth of hyphae, and also to form a robust protein coat on spores. This coating provides a hydrophobic external surface that resists wetting and thus facilitates spore dispersal in air (4–6). The class I hydrophobin rodlets share many of the structural characteristics of amyloid fibrils; formation of the insoluble, fibrillar rodlets is accompanied by conformational change to an ordered cross-β-secondary structure form and the polymerized rodlets, but not the monomeric form of the protein, bind to the dye thioflavin T (ThT)4 (2, 7, 8). However, in contrast to other amyloid fibrils, which can often be solubilized by treatment with denaturants such as guanidine hydrochloride or solvents such as dimethyl sulfoxide (9), treatment with acids such as formic and TFA has been reported to be the only method capable of depolymerizing hydrophobin rodlets and regenerating the monomeric form of the hydrophobin proteins in solution (10, 11). This is similar to the curli and tafi fibrils produced by bacteria, which have been shown to be functional amyloid and which also require treatment with formic acid for dissolution and regeneration of the monomeric, soluble component proteins (12, 13).

The hydrophobins we have used are EAS15 and DewA. EAS15 is a truncated version of EAS, a class I hydrophobin from the bread mold Neurospora crassa, which forms amphipathic rodlets on the spore surface (14, 15). We have determined previously the three-dimensional structures of the monomeric forms of both EAS and EAS15 and have demonstrated that the long Cys3–Cys4 loop is not necessary for rodlet formation or stability (14, 16). EAS15 forms rodlets with very similar properties and kinetics to EAS but has more favorable expression and purification properties. DewA is a 118-residue class I hydrophobin produced by Aspergillus nidulans (17). Immunolocalization studies have shown that the DewA protein is found only on the spore surface, where it contributes to the hydrophobicity of the spore in combination with another class I hydrophobin, RodA (18). Besides the eight cysteines, DewA shares very little sequence similarity with other class I hydrophobins; however, we have been able to express DewA recombinantly in Escherichia coli, and the purified protein spontaneously assembles into rodlets at interfaces.

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2 Supported by an Australian Postgraduate Award.
3 To whom correspondence should be addressed: School of Molecular Bioscience, University of Sydney, NSW 2006, Australia. Fax: 612-93515858; E-mail: margaret.sunde@sydney.edu.au.

□ The abbreviations used are: ThT, thioflavin T; EaN, ethylammonium nitrate; EAS15, truncated version of EAS, with 15 residues removed between Cys3 and Cys4; HHI, hydrophobic:hydrophilic interface; HSQC, heteronuclear single quantum coherence; OTS, octadecytrichlorosilane; TeaAc, triethylammonium acetate.
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There is much interest in the application of amphipathic hydrophobin monolayers in a range of biotechnology applications. Hydrophobins can solubilize and reverse the wettability of nanomaterials such as carbon nanotubes (19–21), be used to functionalize surfaces and be applied for the two-phase separation of proteins (22, 23). Class I hydrophobin monolayers also have been shown to be immunologically inert (24), and together with the ability of hydrophobins to form suspensions with water-insoluble moieties in biological solutions, this has prompted interest in the use of hydrophobins as drug-delivery agents (25, 26). Use of hydrophobins for these purposes requires a detailed understanding of the assembly mechanism so that the process of monolayer formation can be controlled and directed.

We have studied the effect of a number of different solvents and solutes, including alcohols, ionic liquids, and detergents, on rodlet formation and stability to identify the forces that drive this self-assembly. We find the rate of hydrophobin self-assembly is correlated with the surface tension of the solution, and this may offer a practical and reversible way of controlling hydrophobin self-assembly.

EXPERIMENTAL PROCEDURES

Materials—Methanol was purchased from Burdick & Jackson (Muskegon, MI). All other reagents and chemicals were purchased from Sigma-Aldrich, Astral Scientific, Amyl Media, or Ajax Finechem (NSW, Australia).

Production of Recombinant DewA—A synthetic gene encoding the predicted sequence of secreted DewA (signal peptide cleavage site predicted by SignalP (27)) was synthesized (GenScript) and was cloned into the pHUE vector, a kind gift from Dr Rohan Baker (Australian National University) (28). This vector produced recombinant DewA fused to the C terminus of human ubiquitin, with an additional His-tag at the N terminus of the fusion construct. The fusion was overexpressed in E. coli BL21(DE3) cells (Promeqa, Australia) at 37 °C in Luria-Bertani broth after induction with 0.4 mM isopropyl β-D-thiogalactopyranoside (1-thio-β-D-galactopyranoside) and purified under native conditions using nickel-nitrilotriacetic acid-agarose (Qiagen, Hilden, Germany). The fusion protein was dialyzed into a cleavage buffer (20 mM Tris, 50 mM NaCl, 2.5 mM CaCl$_2$, pH 8.0; >6 h), and then the His$_6$-ubiquitin tag was cleaved from DewA by a deubiquitylating enzyme, by incubation at 37 °C for 2 h (28).

The hydrophobin was subjected to reverse phase-HPLC using a C$_{18}$ reverse phase column (Nova-Pak® C18 8 × 100 mm Radial-Pak™, Waters Corp.) for final purification. The protein identity and folding were confirmed by MALDI-TOF/MS and 1H NMR.

Production of Recombinant EAS$_{15}$—EAS$_{15}$ was expressed and purified as described previously (16). The protein identity was confirmed by MALDI-TOF/MS and 1H NMR.

Negative Stain Transmission Electron Microscopy—Fresh samples of EAS$_{15}$ and DewA were prepared from lyophilized protein at a concentration of 0.1 mg/ml in 20% ethanol. Care was taken not to introduce air bubbles when the protein was dissolved. Drops of protein-containing solution (20 µl) were pipetted onto a sheet of Parafilm™ and allowed to stand for 10 min at room temperature. This allowed for the formation of a rodlet monolayer on the drop surface. Rodlets made in the presence of ionic liquid were examined at the end of the ThT experiments. Rodlets treated with water, ethylenimmonium nitrate (EaN), and acetic acid were examined after solubilisation assays. Copper grids (200 mesh from ProSciTech) were prepared with pioloform plastic films and subsequently carbon-coated. Protein was transferred by floating the grid on the surface of the protein-containing drop for 30 s and then washed with water and stained with 2% uranyl acetate for 10 min. Grids were examined in a Phillips CM12 electron microscope operating at 120 kV, equipped with an iTEM digital imaging system, in the Australian Centre for Microscopy and Microanalysis at the University of Sydney.

Contact Angle Measurements—Drops of an aqueous solution of DewA or EAS (20 µl, 0.1 mg/ml) were incubated on octadecyltrichlorosilane (OTS)-coated silicon for 30 min, after which the solution was wicked off, and the surface was air-dried. Water drops (10 µl) were placed onto the protein-covered regions of the OTS-coated silicon. Profiles of the drops were digitized with a contour monitor at room temperature, and the contact angle was obtained using the drop shape analysis software associated with the DSA 10MK2 system (KRÜSS, Hamburg, Germany). Values given are the average from three drops.

Nuclear Magnetic Resonance Spectroscopy—EAS$_{15}$ isotopically labeled with $^{15}$N was expressed and purified as described previously (16). $^{15}$N-labeled EAS$_{15}$ (to a final concentration of ~100 µM) was prepared in 20 mM sodium phosphate (pH 6.0) containing 10% (v/v) D$_2$O and 2 µM 2,2-dimethyl-2-silapentane sulfonylic acid. All $^{1H,^{15}}$N HSQC spectra were recorded at 298 K on a 600-MHz Bruker Avance III spectrometer equipped with a TCI cryoprobe (Karlsruhe, Germany). Aliquots of absolute ethanol were titrated into $^{15}$N-EAS$_{15}$ to a cumulative concentration of 12% (v/v) and a $^{1H,^{15}}$N HSQC spectrum recorded following each addition. NMR data were processed using TOPSPIN (Bruker) and analyzed with SPARKY (University of California, San Francisco). Weighted combined $^{1H}$ and $^{15}$N resonance perturbations (Δδ$_{tot}$) for each assigned residue were calculated using the equation: Δδ$_{tot}$ = ((Δδ$_{HN}$W$_{HN}$)² + (Δδ$_{W}$W$_{W}$))$^{1/2}$, where Δδ is the resonance perturbation and weight factor of nucleus i, respectively, W$_{HN}$ = 1, and W$_{W}$ = 0.154. Previously published NMR $^{1H,^{15}}$N chemical shift assignments for EAS$_{15}$ (BioMagResBank code 15863) were used.

Circular Dichroism Spectropolarimetry—CD measurements with DewA and ionic liquids were performed on an Applied Photophysics Chirascan Spectrometer at 24 °C. The concentration of DewA was 5 µM. Far UV CD spectra with EAS$_{15}$ in water and alcohol solutions were collected on a Jasco 720 spectropolarimeter, at 17, 20, and 80 °C. EAS$_{15}$ concentration was 9.8 µM. All data were baseline-corrected by subtraction of a spectrum of MQW or appropriate solution.

Thioflavin T Binding Fluorescence Assay—All samples for rodlet formation assays were prepared in 2-ml Wheaton glass vials (Sigma). For ionic liquid experiments, lyophilized DewA or EAS$_{15}$ were dissolved in water and added to a solution of ThT in a mixture of an ionic liquid and water, so that the final concentrations in each sample were 32 µM ThT and ~10–14 µM DewA or 4.3 µM EAS$_{15}$. The final concentrations of the ionic liquid in water were 25, 50, or 75% (v/v). For alcohol
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FIGURE 1. The hydrophobins DewA and EAS₁₅ form amphipathic monolayers composed of rodlets. Negatively stained transmission electron micrographs of rodlet monolayers formed by DewA (A) and EAS₁₅ (B). C, images from a video contact angle device showing the side profile of water droplets placed on OTS-treated silicon wafers that had been precoated with DewA or EAS₁₅ proteins or water.

experiments, lyophilized DewA or EAS₁₅ were dissolved in water and added to a solution of ThT in a mixture of an alcohol and water, so that the final concentrations were 40 μM ThT and ~14 μM of DewA or 4.3 μM EAS₁₅. The final proportion of alcohol to water (v/v) was varied and is indicated within the figures. For the preparation of samples in aqueous solutions of detergent, lyophilized DewA, or EAS₁₅ were dissolved in water and added to a solution of ThT in a mixture of Triton X-100 and water, so that the final concentrations were 40 μM ThT, ~14 μM DewA or 3.7 μM EAS₁₅, and either 10⁻⁷ M, 10⁻⁵ M, 10⁻⁶ M, or 10⁻⁸ M of Triton X-100. Samples were agitated at 3000 rpm with a Labnet VX100 Vortex Mixer. Fluorescence was measured using a Varian Cary Eclipse fluorescence spectrophotometer (Varian Inc.). Samples were excited at 435 nm, and fluorescence was recorded from 450–600 nm using excitation and emission slit widths set to 10 nm. The vortexing times shown represent cumulative vortexing time. Intensity at 485 nm was used, and the results are the mean of three replicates.

The extent of rodlet formation was calculated by setting the initial ThT intensity to 0 and scaling the final ThT intensity obtained for each sample to 1 (except for 75% EaN, for which no increase in ThT signals was observed throughout the time course). This allowed for the comparison of the kinetics of rodlet formation between different samples, where different ionic liquids quenched ThT fluorescence to varying extents (see supplemental data).

Measurement of Surface Tension of Ionic Liquid Solutions—Surface tension measurements of the various water:ionic liquid solutions were performed using a CAM 101 goniometer (KSV Instruments, Ltd., Helsinki, Finland) using the pendant drop method. The drop profile was then curve fitted to the Young-Laplace equation using KSV CAM software.

RESULTS

We have been able to express both EAS₁₅ and DewA recombinantly in E. coli. Unlike recombinant EAS₁₅ expressed in E. coli, which is targeted to inclusion bodies and requires oxidative refolding in vitro to acquire the native state (16), DewA remains soluble in the cytoplasm during overexpression and is observed by ¹H NMR to be folded after purification under native conditions. Recombinant DewA and EAS₁₅ assemble spontaneously at air-water interfaces (Fig. 1, A and B). Rodlets assembled on the surface of a water droplet are ~80-nm wide and many hundreds of nm long. The rodlet monolayers are amphipathic and reverse the wettability of surfaces. The contact angles formed by drops of water on a DewA-coated or EAS₁₅-coated OTS-treated silicon wafer are 50.7° ± 4.2 and 56.0° ± 10.9, compared with the angle formed by a water droplet on OTS-treated silicon of 102.4° ± 3.3 (Fig. 1C).

We initially were interested to know whether ionic liquids could be used to dissociate rodlets into the monomeric hydrophobin form. Ionic liquids have attracted much interest recently as solvents for biomolecules because they are non-aqueous solvents that are able to promote the self-assembly of amphiphiles (29), and they appear to stabilize protein function (30, 31), and some can promote, inhibit, or solubilize amyloid fibrils (32–34). To date, only high concentrations of strong acids such as formic acid and TFA have been reported to depolymerize rodlets to the monomeric form (6). With this in mind, we tested the protic ionic liquid triethylammonium trifluoroacetate, which is formed via the neutralization of the base triethylamine and the acid trifluoroacetate, which is formed via the neutralization of the base triethylamine and the acid trifluoroacetate ([(CH₃CH₂)₃ NH₃⁺][CF₃COO⁻]). Rodlets were formed by agitation of the solution and then freeze-dried. Rodlets were resuspended in a neat solution of triethylammonium trifluoroacetate, and these samples were then incubated at room temperature for 16 h. The solution was centrifuged, and the supernatant examined by reverse-phase HPLC to determine whether monomeric hydrophobin had been released into the soluble fraction. However, this neat ionic liquid was not able to dissociate preformed EAS rodlets on this time scale, despite the presence of the trifluoroacetate group. None of the other ionic liquids we tested, including EaN ([(CH₃CH₂NH₃⁺)][NO₃⁻]), ethanolammonium formate ([(HOCH₂CH₂NH₃⁺)][COOH⁻]), triethylammonium acetate (TeaAc, [(CH₃CH₂)₃ NH₃⁺][CH₃COO⁻]), and triethylammonium triflate ([(CH₃CH₂)₃ NH₃⁺][CF₃SO₃⁻]), was able to depolymerize the rodlet form by incubation overnight.
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(Results not shown). Also, morphology of rodlets treated in EaN was unchanged (see supplemental data). Because TFA but not triethylammonium trifluoroacetate was able to dissociate the rodlets, we wondered whether rodlet dissociation is purely caused by the low pH of the acid. Therefore, we next tested the effect of a range of acids on rodlet stability. Concentrated TFA (pKa 0.3) and formic (pKa 3.7) acids were able to readily dissociate preformed EAS15 rodlets as reported previously (6). Neat hydrochloric acid (pKa −8), despite being a much stronger acid than formic or TFA, was noticeably less effective at dissociating EAS15 rodlets. Concentrated acetic acid (a carboxylic acid similar in structure to TFA and formic acid but with a higher pKa of 4.8) was unable to reverse the polymerization; incubation in acetic acid has no effect on rodlet morphology (see supplemental data). These results indicate that a combination of low pH-induced unfolding and the specific nature of the ion are important for reversing rodlet formation. Dimethyl sulfoxide, a solvent which has been used by other groups to dissolve amyloid fibrils (9), does not depolymerize the rodlets but instead, converts a rodlet-containing solution into a gel-like substance (results not shown), possibly because of the effect of the preferential binding of hydrophobic residues to dimethyl sulfoxide (35).

We next determined whether ionic liquids affected hydrophobin self-assembly into rodlets. The rodlets formed by DewA and EAS15 bind the dye ThT and give rise to the characteristic shift in emission spectrum, suggesting that they have a cross-β-core structure, similar to that found in amyloid fibrils and other class I hydrophobin rodlets (7, 14, 36). We have used the increase in ThT fluorescence on binding to rodlets to monitor the rate of rodlet formation. Self-assembly is initiated by vortexing of the solution, which continuously exposes new air: water interfaces at which self-assembly can occur. Addition of ThT to the hydrophobin solution allows the extent of rodlet formation to be monitored. DewA assembly was examined in pure solutions of EaN, TeaAc, triethylammonium trifluoroacetate, and triethylammonium triflate. The effect of EaN concentration on DewA and EAS15 assembly was also studied. Assays were continued for ~10× T1, where T1 is the time constant for the rate of formation of DewA or EAS15 rodlets in water.

No rodlet formation was observed in pure ionic liquid solution over this time course. However, rodlet formation was detected in EaN-water mixtures (Fig. 2A). At 25% EaN, the rate of rodlet formation is slower than that observed in water, and increasing the EaN concentration further diminishes the rate of rodlet formation, until no rodlet formation is observed over 120 min in 75% EaN for either DewA or EAS15 (Fig. 2, B and C). However, rodlets formed in ionic liquid:water mixtures have morphology indistinguishable from those formed in water (see supplemental data). TeaAc had a greater inhibitory effect on DewA self-assembly, with a reduced rate of rodlet formation relative to EaN at 25% ionic liquid (Fig. 2A). Ethanolammonium formate had the least effect on rodlet formation, with rates of rodlet formation in up to 75% ethanolammonium formate being similar to those in water (data not shown).

Performed lyophilized rodlets were not dissolved to any extent in any of the ionic liquids tested over a period of 16 h. It therefore seemed likely that the observed effect of ionic liquids in the rodlet formation assays was an effect on the process of conversion of the monomer into rodlets, rather than a competing solubilization of nascent rodlets. For that reason, we went on to investigate whether the reduced rate of rodlet formation in the presence of ionic liquids could be attributed to a reduction in surface tension at the air:liquid interface, as the trend for individual ionic liquids to inhibit rodlet formation was correlated with the surface tension of the ionic liquid solution. For example, rodlet formation was slowed but still observed in 50% EaN, but no rodlet formation was observed over 10× T1 in solutions of 75% EaN. The surface tension in these two solutions is 50 and 47 mN m⁻¹ for 50% EaN and 75% EaN, respectively, at 24°C (Table 1.). The reduction in rodlet formation was observed at lower concentrations of TeaAc, for example, no rodlets were formed over the time course in a solution containing 50% TeaAc, which has a surface tension of 41 mN m⁻¹,
whereas rodlet formation was observed in a solution containing 75% ethanolammonium formate, which has a surface tension similar to water.

To investigate this idea further, we measured rodlet formation by DewA and by EAS$_{315}$ in alcohol:water mixtures that would allow us to tune the surface tension of the solution without large changes in the nature of the solution or the additive. In alcohol:water mixtures, there is a rapid decrease in the surface tension of the bulk solution with the addition of relatively small percentages of alcohol. Experiments were carried out to determine the amounts of ethanol, methanol, and 1-propanol required to reduce the rate of rodlet formation by EAS$_{315}$ or DewA (Fig. 3, A–D). It was found that addition of 13% methanol prevented EAS$_{315}$ rodlet formation completely for at least $10 \times T_{1EAS_{315}}$, whereas addition of only 10% ethanol and 3% 1-propanol had the same effect on this protein. Similarly, addition of ethanol significantly reduced the rate of DewA rodlet formation, but in this case, ~15% ethanol was required to eliminate rodlet assembly for at least $10 \times T_{1DewA}$. These results are consistent with the reduction of surface tension of the solution on addition of these alcohols (Fig. 3E). Fig. 3E was prepared from data published by Vazquez et al. (37) and shows a comparison of the surface tension of solutions of these alcohols in water, and it can be seen that when the surface tension of the solution falls below ~54 mN m$^{-1}$, EAS rodlet formation is not observed on this time scale, regardless of the nature of the alcohol addition. For DewA, this threshold appears to be lower at ~43 mN m$^{-1}$.

TABLE 1
Surface tension of ionic liquid solutions (mN m$^{-1}$)

| Ionic liquid | 0%  | 25%  | 50%  | 75%  | 100% |
|--------------|-----|------|------|------|------|
| EaN          | 70 ± 2.2 | 55 ± 2.3 | 50 ± 2.2 | 47 ± 2.3 | 42 ± 2.2 |
| TeaAc        | 70 ± 2.2 | 46.5 ± 2.3 | 41 ± 2.3 | 38 ± 2.3 | 34 ± 2.2 |

FIGURE 3. Alcohols reduce the extent of rodlet formation in a manner consistent with the reduction in surface tension. Solutions containing hydrophobin, ThT, and various alcohols were agitated at 3000 rpm for varying lengths of time. A, EAS$_{315}$ + ethanol; B, EAS$_{315}$ + 1-propanol; C, EAS$_{315}$ + methanol; and D, DewA + ethanol. Error bars shown are one S.D. from the mean of three replicates. E, graph showing the relationship between surface tension and varying concentration of ethanol, methanol, and 1-propanol in water using published data (37). Arrows and gray shading indicate the approximate surface tension corresponding to alcohol concentrations above which no EAS$_{315}$ or DewA rodlet formation was observed in a period up to $10 \times T_{1}$, where $T_{1}$ is the time constant for the rate of self-assembly of the corresponding protein in water.
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FIGURE 4. Detergent reduces the extent of rodlet formation in a manner consistent with the reduction in surface tension. Solutions containing hydrophobin, ThT, and Triton X-100 were agitated at 3000 rpm for varying lengths of time. A, EAS_{15}; B, DewA. Error bars shown are one S.D. from the mean of three replicates.

Significantly, when the alcohol was removed by lyophilization, and the protein was redissolved in water, the protein could assemble into rodlets at the expected, uninhibited rate (results not shown). This demonstrates there is no irreversible alteration to the assembly properties of the protein.

The nonionic detergent Triton X-100 also slows hydrophobin rodlet assembly in a manner correlating with its effect on surface tension (Fig. 3D). The experiments were performed with Triton X-100 concentrations below the critical micelle concentration (critical micelle concentration for Triton X-100 is \( \sim 2.4 \times 10^{-5} \) M) but over a range that resulted in reduction of the surface tension of the solution from \( \sim 68 (10^{-5} \) M) to \( \sim 38 \) mN m\(^{-1}\) (\(10^{-4} \) M), Surface tension of Triton X-100 solutions were obtained from Ref. 38. The same trend of decreasing rate of rodlet assembly with decreasing surface tension of the solution was observed. For EAS_{15}, when the surface tension was reduced to \( \sim 65 \) mN m\(^{-1}\) by inclusion of \(10^{-6}\) M Triton X-100, the rate of rodlet formation was reduced only slightly. However, when the surface tension was reduced to \( \sim 52 \) mN m\(^{-1}\) in a solution containing \(10^{-5}\) M Triton X-100, no EAS_{15} rodlet assembly could be detected in a time period corresponding to \( 10 \times \) time period corresponding to \( 10 \times T1_{EASA_{15}} \) (Fig. 4A). As observed with the alcohol additions, a lower surface tension is required to significantly slow DewA rodlet formation (between \(10^{-5}\) and \(10^{-4}\) M Triton X-100, corresponding to surface tension between \( \sim 52 \) and \(38 \) mN m\(^{-1}\); Fig. 4B). These experiments demonstrate that hydrophobin rodlet formation can be slowed by lowering of surface tension but that individual hydrophobins are affected to different extents by different solvents, possibly because of differing protein sequences, levels of amphipathicity and total exposed hydrophobic surface areas. These differences are highlighted by the fact that the rates of EAS_{15} and DewA rodlet assembly differ significantly, even in the same solvent.

Although additives can affect the viscosity of the solutions, over the range of additive concentration, we have tested the viscosity of the solutions was not increased beyond 1.8 times the viscosity of water. Viscosity in the range \(10\times\) that of water begins to have a significant effect on bubble size when streams of bubbles are introduced into a solution (39, 40). Our assay was designed to continuously expose new air:water interfaces, and thus, given the small increases in viscosity, it is unlikely that the large reduction in the rate of rodlet formation is due to insufficient availability of air:water interface.

The effect on rodlet formation of ionic liquids and alcohols observed here was not due to gross disruption or destabilization of native protein conformation by these solutes. The effect of ionic liquids and alcohols on the conformation of the proteins was studied by far UV circular dichroism spectropolarimetry and NMR spectroscopy. Only CD data could be collected for ionic liquid solutions because the high ionic strength of these solutions made it impossible to collect quality NMR spectra from them. The far UV CD spectra of soluble DewA in ionic liquid solutions and EAS_{15} in alcohol solutions indicate that these solutions do not have a dramatic effect on the conformation of the proteins within the bulk solution (Fig. 5, A and B). The small but significant differences may arise from changes in the unstructured regions of the protein. To examine these changes in more detail, a \(^{15}\)N HSQC titration study into the effect of addition of ethanol into \(^{15}\)N-labeled EAS_{15} was carried out. This allows a residue-level investigation of the effects of ethanol addition on protein structure. It shows only minor changes in the positions of the amide peaks across the spectra (Fig. 5C). Furthermore, the amount and direction of peak position changes is relatively uniform across the entire spectrum, even up to 12% ethanol. In particular, the relative positions of the peaks that arise from the structured regions of the protein remain essentially the same, indicating the structured regions of the protein remain in a similar conformation over the concentrations of alcohol tested. This suggests the small general peak changes result from differences in solvent properties (e.g., pH, amide exchange rate with water molecules) rather than a gross change in protein conformation.

To determine whether the observed reduction in rodlet formation rate in the presence of additives is a result of protein destabilization, we recorded CD spectra of EAS_{15} in the absence and presence of 20% ethanol at 20 and 80 °C. The spectra are essentially identical, indicating no major unfolding of the hydrophobin structure occurs at temperatures up to 80 °C, regardless of solvents (see supplemental data).

DISCUSSION

The reduction in observed rate of rodlet formation on addition of additives was correlated with the surface tension of the solution rather than with the absolute amount or the nature of the additives. Although it is conceivable that these additives could slow rodlet formation by disrupting or destabilizing protein structures, this does not appear to be the case for hydrophobin rodlet assembly. There was no significant change to the
structures or stability of the hydrophobin monomers in the presence of additives, as shown by the CD and NMR data, suggesting that the reduction in assembly rate is likely to be a consequence of the change in bulk solvent properties. Increased surface tension is correlated with exclusion of solute from the surface and vice versa (41, 42), and aqueous alcohols and ionic liquids are surface-adsorbed solutes (43). Class I hydrophobins are amphipathic and migrate preferentially to a hydrophobic:hydrophilic interface (HHI) because they can satisfy the requirement to form hydrogen bonds with water on one face only (1, 44). Although the results presented here do not provide direct evidence for a competition between protein and co-solutes at the air:water interface, it is possible that adsorption of the solutes at the surface may result in exclusion of the protein from the interface to some extent and may contribute to reduction in rodlet assembly (Fig. 6A).

In addition, solutes that are preferentially adsorbed at the air:solution interface can also be preferentially adsorbed onto a protein surface in an aqueous environment, as this represents another HHI (Fig. 6A). This phenomenon may be particularly applicable to hydrophobins, which have relatively large exposed surface hydrophobic areas. The adsorption/interaction of solute molecules to hydrophobic areas may result in reduced interaction of the hydrophobins with the air:solution interface, thereby reducing rodlet assembly.

Given the self-assembly of hydrophobin rodlets only occurs at a HHI, this raises an interesting question as to the underlying molecular mechanisms of rodlet assembly. One explanation is that the local increase in hydrophobin concentration brought about by migration to the surface may be necessary to trigger self-assembly into rodlets. Other work with amyloidogenic peptides that are surface-active has shown that the HHI both concentrates the amphiphilic peptides and promotes peptide alignment which favors intermolecular assembly through β-sheet formation (45, 46). This all combines to make nucleation many orders of magnitude faster at HHIs like the air:water interface. Conversely, the formation of a layer enriched in ordered additive molecules may act to reduce the concentration and alignment of amphipathic proteins such as hydrophobins at the surface, eventually to a level that cannot support nucleation and thus self-assembly (Fig. 6A).

However, although other surface-active peptides such as islet-associated polypeptide will still associate to form amyloid fibrils in solution in the absence of an air:water interface when a critical concentration is exceeded (1.8 M for islet-associated polypeptide) (45), class I hydrophobins have not been found to self-assemble in the absence of a HHI, even at high concentrations. In our experience, a layer of rodlets readily forms at the air:water interface in unvortexed solutions of class I hydrophobins, but the bulk of the protein remains monomeric, even at mM concentrations. Only when the protein solution is agitated, thus continuously introducing new air:water interfaces, can most of the protein in solution be converted into the rodlet.
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β-sheet-rich assembled form. This suggests the role of the HHI in rodlet formation must extend beyond its effect on local protein concentration and also must be associated with the ability of the hydrophobin to undergo the specific conformational conversion and intermolecular association that exposes the amyloidogenic region and results in a cross-β-amyloid rodlet (Fig. 6B).

“Surface denaturation” is a recognized and well studied phenomenon, one that is particularly important, given the trend toward using proteins adsorbed onto surfaces as biosensors and the requirement for maintenance of native structure in a thin film on a substrate. When folded proteins that have buried hydrophobic regions reach a hydrophobic interface, the hydrophobic regions are drawn toward the interface, and the proteins undergo a conformational change to maximize the interactions with the hydrophobic surface. This can have the consequence of partially or completely unfolding the protein (47). Several proteins have been shown to undergo conformational change at the interface and to become unfolded into flat peptide sheets (48). Both the Aβ peptide and islet-associated polypeptide of Alzheimer disease, the peptide that forms fibrils in Type 2 diabetes, have been shown to readily adsorb to and form β-sheets at HHIIs. Recently, Hoernke and colleagues (49) have demonstrated that a surface-active peptide designed to be able to form both coiled-coil and β-sheet structures undergoes a time- and concentration-dependent conversion into a stable β-sheet form in a way that is influenced strongly by the HHI. The influence of solvent properties on protein interactions and/or protein conformation changes at interfaces is likely to be applicable to other surface active proteins in addition to hydrophobins.

Although surface-driven conformational change/denaturation resulting in pathological amyloid formation is unintentional, rodlet formation by class I hydrophobins may represent an unusual application of the same phenomenon by nature. For this class of functional amyloid-forming proteins, this surface “denaturation” or alteration of the native, soluble fold may be the basis for the conversion of class I hydrophobins into an assembly-competent form. Therefore, an additional means through which the additives may act to decrease rodlet formation is by reducing the drive for the hydrophobin molecules to undergo conformational changes that are required for rodlet assembly, for example, by providing compensating interactions with the exposed hydrophobic regions.

This study has demonstrated that the rate of hydrophobin rodlet formation is correlated with the surface tension of the solution and can be slowed by the addition of ionic liquids, alcohols, and detergents. The ability to control the rate of rodlet formation may be useful as the biotechnological potential of hydrophobins is developed. Our results allow us to propose that hydrophobin rodlet formation is driven by the properties of a HHI and occurs in at least three stages (Fig. 6): (i) an initial concentration and alignment of the hydrophobins at the HHI, (ii) a conformational rearrangement of the protein chain to maximize the favorable interactions with the hydrophobic surface, and (iii) associated conformational change that allows intermolecular interactions (including H-bonding) to generate the cross-β-core of the rodlets.

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