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# Spectroscopic Evidence for Amyloid-like Interfacial Self-Assembly of Hydrophobin Sc3

Peter Butko,* Justin P. Buford,*,[1] J. Shawn Goodwin,* Paul A. Stroud,† Charles L. McCormick,† and Gordon C. Cannon*

**Department of Chemistry & Biochemistry and †Department of Polymer Science, University of Southern Mississippi, Hattiesburg, Mississippi 39406*

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**Amphipathic fungal proteins called hydrophobins are able to self-assemble into insoluble supramolecular structures at hydrophobic/hydrophilic interfaces, but the molecular mechanism and underlying protein conformation changes are not known. Secondary-structure prediction indicated that hydrophobin Sc3 is an all-β protein. Many amyloidogenic proteins self-assemble into insoluble amyloid fibrils while undergoing a change to an all-β conformation. In this study we show that two dyes, thioflavin T, and Congo red, which are widely used for specific detection of stacked β sheets, interact with Sc3 assemblies in the same way as with the amyloid β-sheet fibrils. We conclude that Sc3, and probably other hydrophobins too, self-assemble at interfaces in the same manner as amyloidogenic proteins, i.e., through β-sheet stacking.** 

*Key Words:* **hydrophobin; protein self-assembly; amyloid; β-sheet stacking; thioflavin T fluorescence; Congo red.**

Hydrophobins are small, about 100 amino acids long, amphipathic fungal proteins that exhibit the remarkable property of self-assembling into insoluble membranes on hydrophobic/hydrophilic interfaces (1, 2). Because of this ability, hydrophobins offer a wide range of possible applications in technology. They adhere to both hydrophilic and hydrophobic surfaces, such as glass, mica, Parafilm, and Teflon, thereby modifying their properties (2–5). Furthermore, they self-assemble on the surface of microscopic gas bubbles upon vortexing or gas-bubbling the protein solution (3) and on the surface of oil droplets in water (2). The latter phenomenon has been shown to lead to efficient hydrocarbon sequestration and stabilization of aqueous oil emulsions (6).

A coherent picture of the biological significance of hydrophobins formed just recently. Hydrophobin assemblies have been found on the surface of the fungal aerial hyphae, filamentous reproductive structures that emerge from aqueous environment into the air (7). Since hydrophobins are surface-active—they decrease the surface tension of water from 72 mN/m to 43 or 32 mN/m, values characteristic for synthetic surfactants (8, 9)—these proteins may aid the hyphae to break through the air/water interface and protect them against dehydration (10). In addition, hydrophobins may facilitate adhesion of pathogenic fungi to hydrophobic surfaces on a host organism, such as the plant leaf or insect cuticle (2).

Little is known about the hydrophobin self-assembly mechanism at the molecular level. The amino-acid sequences of all known hydrophobins show 34% similarity and exhibit two common characteristic features: the presence of a short signal sequence predestining the protein for secretion and the presence of eight cysteine residues whose relative positions are conserved (1). In fact, the pattern -C-$X_{5–9}$-C-C-$X_{6–39}$-C- (where C and X denote cysteine and any amino acid, respectively), present in both the N- and C-terminal halves of the molecule, is considered a signature of hydrophobins. Four disulfide bridges have been found in one hydrophobin, cerato-ulmin: C1-C2, C3-C4, C5-C6, and C7-C8 (11). Without proof, this pattern has been tentatively assumed for all hydrophobins. The importance of the disulfide bridges for the protein self-assembly is not clear, but hydrophobin assemblies can be dissolved by trifluoroacetic acid treatment in the absence of reducing agents (12) and hydrophobin is still capable of self-assembly after covalent labeling with a sulfhydryl-reactive fluorescence probe 5-((((2-iodoacetyl)amino)-ethyl)amino)naphthalene-1-sulfonic acid (1,5-IAEDANS) (Goodwin, J. S., Cannon, G. C., and Butko, P., unpub-

Abbreviations used: Aβ, amyloid β peptide (1-40); CR, Congo red; ThT, thioflavin T.

[1] Participant in the University of Southern Mississippi Summer Undergraduate Research Program. Permanent address: Department of Chemistry, Delta State University, Cleveland, MS 38733.

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lished results). These and other data indicate that the disulfide bridges are not essential for self-assembly (13).

So far, no 3D structure has been determined for any hydrophobin. We submitted the Sc3 sequence to a profile-fed neural network system in European Molecular Biology Laboratory at Heidelberg, Germany (14), which predicted that the hydrophobin Sc3 contains 48% $\beta$ strands (6 or 7 strands), 52% loops, and no $\alpha$ helix. This is in accord with the circular dichroism (CD) data (9).

The high content of $\beta$ strands in Sc3 and the exceptional stability of the Sc3 assemblies (they are not soluble in boiling sodium dodecyl sulfate) are the two properties that Sc3 shares with many proteins that form insoluble $\beta$-sheet stacks called amyloid fibrils. The most notorious of this class of proteins is amyloid-$\beta$ peptide (15), but there is a long list of unrelated proteins that are amyloidogenic (16). We therefore put forward the hypothesis that Sc3 may self-assemble via $\beta$-sheet stacking. To test the hypothesis, we employed two spectroscopic methods for detection and quantitation of $\beta$-sheet stacking. Thioflavin T (ThT) is known to greatly increase its fluorescence yield upon binding to stacked $\beta$-sheets (16) and Congo red (CR) exhibits a significant shift in its absorption spectrum in the presence of stacked $\beta$-sheets (17). Both methods are considered highly specific for detecting stacked $\beta$ sheets, as opposed to single $\beta$ sheets or strands: neither ThT nor CR exhibit spectral changes in the presence of proteins that have little or no $\beta$ structure or proteins that aggregate by means other than $\beta$-sheet stacking. This work represents a thorough, controlled study of binding between ThT or CR and Sc3, complementing and extending the preliminary observation of de Vocht *et al.* (13), which appeared during the preparation of our manuscript.

## MATERIALS AND METHODS

For this study we have chosen the hydrophobin Sc3 from *Schizophyllum commune* since it is the best characterized hydrophobin to date. The fungus, purchased from American Type Culture Collection (Rockville, MD), was grown and the protein was purified as described previously (5). Bovine serum albumin (BSA), the 40 amino acid long amyloid $\beta$ peptide 1–40 (A$\beta$), ThT and CR were from Sigma-Aldrich (St. Louis, MO); salts, buffers and other chemicals were from Fisher or VWR (Suwanee, GA); recombinant apolipophorin III (Apo-III) was purified as described (18). Spectra were measured with a Hitachi F-2000 (Tokyo, Japan) or an Edinburgh Instruments FS/FL 900 CDT (Edinburgh, Scotland) spectrofluorometers and a Beckman DU-640 UV/Vis spectrophotometer (Fullerton, CA). The assays were performed at room temperature exactly as described (16, 17).

## RESULTS AND DISCUSSION

First, we wanted to test if ThT was responsive to Sc3 self-assembly. Figure 1 shows that ThT fluorescence increased 8-fold upon mixing with the Sc3 preassembled by vortexing. A smaller, 4.5-fold increase was observed with Sc3 that was not vortexed. This indicates that the protein, which had been kept as 1.5 $\mu$M stock in the refrigerator, was not completely monomeric before the assay or, alternatively, the protein contains some stacked $\beta$ sheets even in the monomer form. We note that Sc3 is 2.5 times larger than the archetypal molecule of amyloid aggregation, A$\beta$, which is disordered in the monomer form. According to the secondary structure prediction, mentioned above, Sc3 contains 6 or 7 $\beta$ strands that may form a $\beta$-sheet system recognized by ThT even in the monomeric protein.

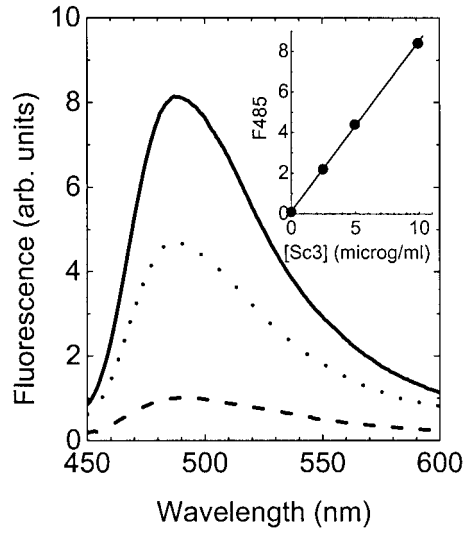


**FIG. 1.** Fluorescence emission spectra of thioflavin T in the presence of Sc3. The sample contained 5 $\mu$M ThT in a 50 mM glycine–NaOH buffer, pH 8.5, either alone (dashed line) or in the presence of vortexed (solid line) or unvortexed (dotted line) 0.7 $\mu$M Sc3. Excitation wavelength was 435 nm, the excitation and emission slits were 5 nm. The inset shows dependence of the fluorescence intensity at 485 nm on the concentration of Sc3 assemblies expressed in $\mu$g/ml.

Since fluorescence of ThT depends on the number of binding sites, affinity and quantum yield, the fluorescence method cannot provide absolute concentration of assemblies. However, it is important to demonstrate that ThT fluorescence specifically and uniquely responds to the amount of $\beta$-sheet assemblies in the sample. As is shown in the inset of Fig. 1, fluorescence is, indeed, directly proportional to the concentration of assembled Sc3. To further ascertain the specificity of the method, ThT fluorescence was measured in the presence of three other proteins: A$\beta$, BSA and apolipophorin III. A$\beta$ served as a positive control since this peptide is well known to self-assemble into long $\beta$-sheet fibrils after prolonged incubation in aqueous solutions. The other two proteins served as negative controls: serum albumin only contains little $\beta$ structure (19), if any (20), while Apo-III contains none (21), and neither is known to assemble via $\beta$-sheet stacking. Results, summarized in Table 1, show that ThT fluorescence in the presence of Sc3 and A$\beta$, but not BSA or

Apo-III, increased several fold upon the proteins' self-assembly. BSA showed some propensity to interact with ThT, but, importantly, ThT fluorescence did not change upon vortexing (Table 1) or prolonged incubation (not shown). These data demonstrate that the self-assembly of Sc3 shares features with that of A$\beta$.

In order to independently confirm the results obtained with ThT we employed the Congo red method, which is based on a shift in absorption spectra caused by binding of CR to ordered stacks of $\beta$ sheets. Although molecular details of the interaction between stacked $\beta$ sheets and the dyes ThT and CR are lacking, it is generally assumed that, due to their opposite charges, the two dyes do not bind to the same sites on proteins. Nevertheless, the spectral changes of both dyes are uniquely dependent on binding to stacked $\beta$ sheets. Changes in CR spectra upon interaction with A$\beta$ are well documented in the literature (17), but Sc3 produced a hardly discernible effect (data not shown). Since both CR and Sc3 are negatively charged at neutral pH (the pI of Sc3 was calculated as 4.35), it is possible that electrostatic repulsion prevented the dye from binding. We therefore tested if the CR method would work at a lower pH. Preliminary experiments probing surface hydrophobicity as a function of pH indicate that Sc3 does not undergo gross conformational changes between pH 7.5 and 4 (Goodwin, J. S., Cannon, G. C., and Butko, P., unpublished results). Difference spectra (protein/CR complex-CR alone) of the Sc3 and A$\beta$ assemblies in complex with CR, recorded at pH 4, are in Fig. 2. Although Sc3 shows a smaller signal than A$\beta$, similarity of the two composite bands at 510 nm is obvious. BSA and Apo-III did not produce appreciable difference spectra (not shown). Thus, at low pH, Congo red is able to bind to stacked $\beta$ sheets in Sc3 and these data confirm the results obtained with ThT.

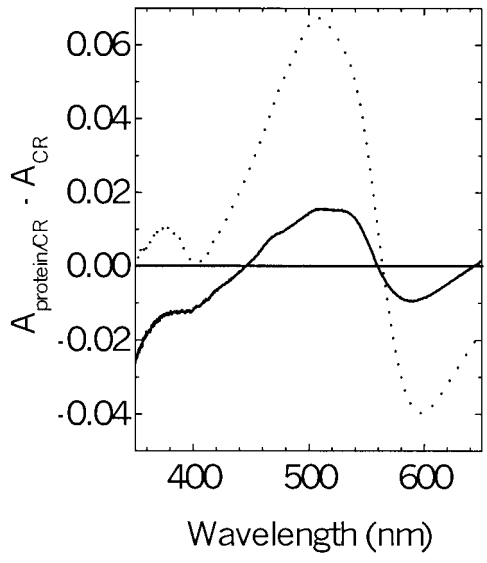


**FIG. 2.** Difference absorption spectra of Congo red in the presence of Sc3 or A$\beta$. Spectrum of Congo red was subtracted from the spectra of the protein/Congo red complex corrected for light scattering. Sample contained 6 $\mu$M CR and 1 $\mu$M vortexed Sc3 (solid line) or 0.6 $\mu$M assembled A$\beta$ (dotted line) in a 50 mM citrate buffer, pH 4.

**TABLE 1**

Spectral Characteristics of Thioflavin T in the Presence of Sc3 and the Control Proteins

| | F485 | |
|---|---|---|
| Protein | Before assembly | After assembly |
| Sc3 | 4.3 ± 1.4 | 9.4 ± 1.2 |
| A$\beta$ | 3.9 ± 1.2 | 11.9 ± 2.7 |
| BSA | 2.9 ± 0.1 | 2.6 ± 0.1 |
| Apo-III | 1.7 | 1.6 |

*Note.* The values are ThT fluorescence intensities at 485 nm normalized to the fluorescence of the dye alone. For Sc3 and BSA the self-assembly (if any) was induced by vortexing for 2 min, for A$\beta$ by incubation for 5 weeks at room temperature (vortexing of A$\beta$ caused too great an increase in the sample turbidity). Protein concentrations in the cuvette were (in $\mu$g/ml): 14 for Sc3 and BSA, 9 for Apo-III and 2.4 for A$\beta$. The experiments were performed 3 times with independent protein preparations, with the exception of Apo-III which was measured only once; the average values ± SEM are given in the table.

Our results strongly indicate that hydrophobin Sc3 self-assembles at water/air interface through $\beta$-sheet stacking, a mechanism operating in amyloidogenic proteins. This conclusion is based on results of two independent spectroscopic techniques known to be very specific for detection of ordered $\beta$-sheet stacks and is greatly strengthened by the data obtained with judiciously chosen control proteins, the amyloidogenic A$\beta$ as a positive control and the non-amyloidogenic BSA and apolipophorin III as negative controls. Additional support for our hypothesis can be found in the published literature: at the wavelengths below 215 nm, CD spectra of Sc3, measured by de Vocht *et al.* (9, 13), clearly show a red shift of about 5 nm after self-assembly at the air/water interface. Such red shifts in $\beta$-sheet CD spectra have been attributed to $\beta$-sheet stacking in the amyloid fibrils of amyloidogenic proteins (22).

In conclusion, the present results provide an easy, sensitive and reliable method for monitoring the degree or extent of hydrophobin self-assembly. Even more importantly, our finding represents an important step towards elucidating molecular mechanism of the interfacial self-assembly of hydrophobins.

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