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# Structural and Functional Role of the Disulfide Bridges in the Hydrophobin SC3*

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**Hydrophobins function in fungal development by self-assembly at hydrophobic-hydrophilic interfaces such as the interface between the fungal cell wall and the air or a hydrophobic solid. These proteins contain eight conserved cysteine residues that form four disulfide bonds. To study the effect of the disulfide bridges on the self-assembly, the disulfides of the SC3 hydrophobin were reduced with 1,4-dithiothreitol. The free thiols were then blocked with either iodoacetic acid (IAA) or iodoacetamide (IAM), introducing eight or zero negative charges, respectively. Circular dichroism and infrared spectroscopy showed that after opening of the disulfide bridges SC3 is initially unfolded. IAA-SC3 did not self-assemble at the air-water interface upon shaking an aqueous solution. Remarkably, after drying down IAA-SC3 or after exposing it to Teflon, it refolded into a structure similar to that observed for native SC3 at these interfaces. Iodoacetamide-SC3 on the other hand, which does not contain extra charges, spontaneously refolded in water in the amyloid-like β-sheet conformation, characteristic for SC3 assembled at the water-air interface. From this we conclude that the disulfide bridges of SC3 are not directly involved in self-assembly but keep hydrophobin monomers soluble in the fungal cell or its aqueous environment, preventing premature self-assembly.**

Hydrophobins are small, moderately hydrophobic proteins characterized by similar hydropathy patterns and the presence of eight cysteine residues with conserved spacing. They fulfill a broad spectrum of functions in fungal development (1). Hydrophobins are secreted by filamentous fungi into their aqueous environment, where they self-assemble at the interface between the medium and the air. This results in a dramatic drop of the water surface tension, enabling fungi to breach the interface to form aerial reproductive structures (2). Hydrophobins also provide these aerial structures with a hydrophobic coating (1) and mediate attachment of fungi to hydrophobic surfaces (for example, in pathogenic interactions) (3, 4).

SC3 of *Schizophyllum commune* is the best studied hydrophobin, but other hydrophobins show similar properties. SC3 is a glycoprotein of 112 amino acids containing 16–22 mannose residues that are probably linked to 12 threonines and 1 serine at the amino-terminal part of the molecule (5). SC3 is a remarkable protein because it forms an insoluble amphipathic protein film after self-assembly at a hydrophobic-hydrophilic interface that cannot be dissociated by heating in 2% SDS at 100 °C (6). The hydrophobic side of this protein film is characterized by a mosaic of parallel rodlets (6) of an amyloid-like nature.[1] Upon self-assembly, the different conformations of SC3 are observed depending on the nature of the interface. For instance, after binding to a hydrophobic solid the protein is trapped in a so-called "α-helical state" (5). With the proper treatment this state can be converted to the β-sheet state,[2] which is also found after assembly on the air-water interface (5). All hydrophobins studied so far have the same spatial distribution of cysteine residues, which is as follows: $X_{2-38}$-C-$X_{5-9}$-C-C-$X_{11-44}$-C-$X_{8-23}$-C-$X_{5-9}$-C-C-$X_{6-18}$-C-$X_{2-14}$.

The initial idea that intermolecular disulfide bridges were formed during the self-assembly and thus stabilize the protein film was disproved when it was shown that SC3 self-assembled not only on air bubbles but also on non-oxidative nitrogen gas bubbles (6). Moreover, it was shown that monomeric SC3 contained no free cysteine residues and that the protein film could be disassembled by treatment with trifluoroacetic acid in the absence of reducing agent (7).

SC3 is a so-called class I hydrophobin (1). The arrangement of the disulfide bridges has been determined for the class II hydrophobin cerato-ulmin (8). Cys[1] is bonded to Cys[2] or Cys[3]; Cys[2] or Cys[3] is bonded to Cys[4]; Cys[5] is bonded to Cys[6] or Cys[7]; and Cys[6] or Cys[7] is bonded to Cys[8]. This results in a primary structure with four loops and two domains. Although there is no direct proof, the general assumption is that a similar pattern of disulfide bridges exists in SC3 (Fig. 1).

Disulfide bonds can fulfill diverse functions in proteins. In general they stabilize proteins, but the exact manner by which the stabilizing effect is achieved cannot be predicted adequately by current theories (9). The conserved pattern of the cysteine residues in all hydrophobins suggests that the cysteine residues are important for hydrophobin structure and/or function. To study the role of the disulfide bridges in SC3, all disulfides were reduced with DTT,[3] and the free cysteines were blocked with iodoacetic acid or iodoacetamide. The secondary structure of carboxymethylated SC3 before and after the self-assembly processes was determined. The strength of binding to

[1] M. L. de Vocht, I. Reviakine, H. A. B. Wösten, A. Brisson, J. G. H. Wessels, and G. T. Robillard, manuscript in preparation.

[2] M. L. de Vocht, I. Reviakine, H. A. B. Wösten, A. Brisson, J. G. H. Wessels, and G. T. Robillard, manuscript in preparation.

[3] The abbreviations used are: DTT, 1,4-dithiothreitol; IAA, iodoacetic acid; IAM, iodoacetamide; ThT, thioflavin T.

FIG. 1. **Amino acid sequence of SC3 and its putative disulfide bridges.** The cysteine residues are indicated in *black*, and the predicted glycosylation sites are indicated in *gray* (5). All hydrophobins are characterized by the same pattern of cysteine residues.
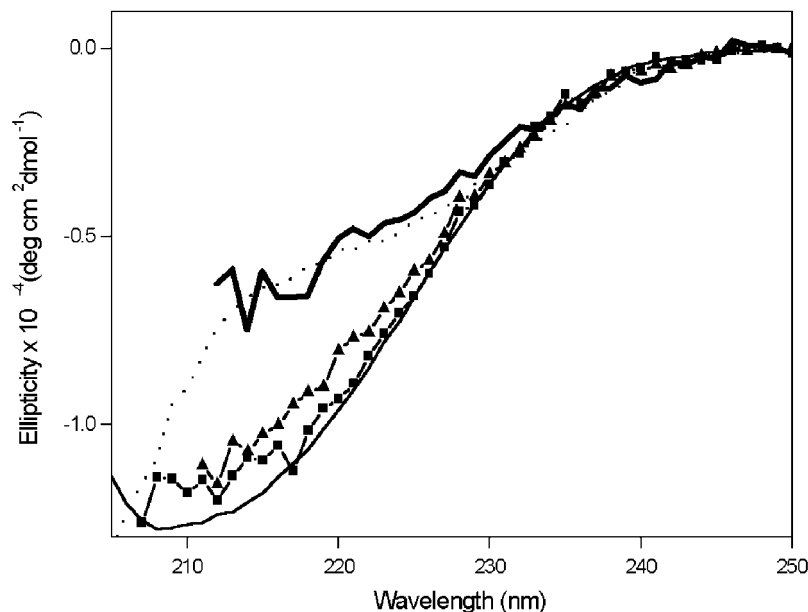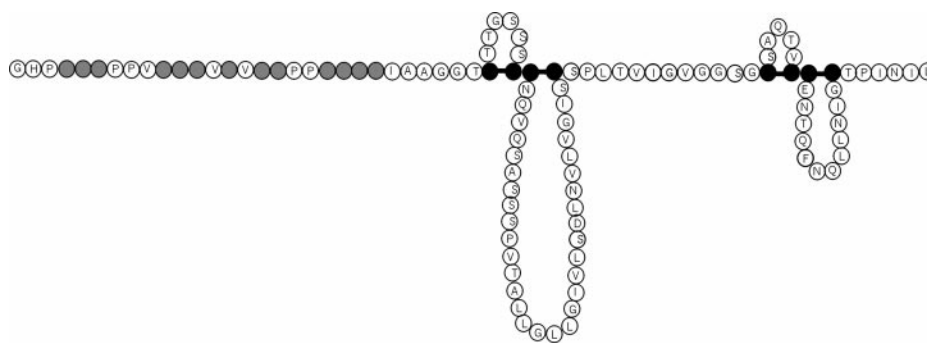


FIG. 2. **Circular dichroism spectra between 212 and 250 nm of monomeric SC3.** Spectra of monomeric SC3 in 50 mM phosphate (*thin line*) and after addition of 10 mM DTT (*line with squares*) or 5.4 M guanidine (*line with triangles*) or both 10 mM DTT and 5.4 M guanidine. The protein unfolded is indicated by a *thick line*. The same spectrum was found for IAA-SC3 (*dotted line*).

a hydrophobic Teflon surface and the ability to self-assemble in the amyloid-like form and to form rodlets were also monitored.

### EXPERIMENTAL PROCEDURES

*Protein*—The hydrophobin SC3 was purified from the culture medium of strain 4-40 of *S. commune* (CBS 340.81) as described (1, 6, 10). Before use, the freeze-dried SC3 was disassembled with pure trifluoroacetic acid and dried in a stream of nitrogen. The monomeric protein was then dissolved in the desired buffer. SC3 was labeled with [$^{35}$S]sulfate as described (11, 12).

*Carboxymethylation of SC3 with Iodoacetic Acid or Iodoacetamide*—Reduction of SC3 and carboxymethylation of the free cysteine residues were performed essentially as described by Hollecker (13). 1 mg of SC3 was incubated for 30 min in 0.5 ml of buffer containing 75 mM Tris/HCl (pH 8.0), 5.4 M guanidine hydrochloride, 2.5 mM EDTA, and 1 mM DTT at 37 °C. This was followed by adding 50 µl of 0.2 M iodoacetic acid (IAA) or 0.2 M iodoacetamide (IAM) in 75 mM Tris (pH 8.0) and incubating the mixture for 15 min at room temperature. After the reaction the sample was dialyzed exhaustively against water and lyophilized.

*Mass Analysis*—Matrix-assisted laser desorption-ionization mass spectrometry was used to follow the reaction of SC3 with IAM and IAA. 5 µl of protein solution (~100 µg/ml) was mixed with 5 µl of matrix (20 mg/ml sinapinic acid in acetonitrile/0.1% trifluoroacetic acid (40/60, v/v)), and 2 µl of the mixture was dried on a target. Spectra were recorded on a TofSpec E and SE micromass spectrometer.

*Secondary Structure Measurements*—The secondary structure of the carboxymethylated SC3 was studied with circular dichroism spectroscopy (CD). The CD spectra were recorded over the wavelength region 190–250 nm on an Aviv 62A DS CD spectrometer using a 1-mm quartz cuvette. The sample compartment was continuously flushed with nitrogen gas, and the temperature was kept constant at 25 °C unless stated otherwise. 10 scans were averaged using a bandwidth of 1 nm, a stepwidth of 1 nm, and 1-s averaging per point. The spectra were corrected using a reference solution without the protein. Typically a protein concentration of 10 µM in 20 mM phosphate (pH 7.0) was used.

To obtain spectra of the protein assembled on the air-water interface, the solution was vigorously shaken for 2 min. For spectra of SC3 bound to a hydrophobic support, colloidal Teflon was added to the solution. No scattering artifacts were observed for the colloidal Teflon, and the same procedure was used as described earlier (5).

To determine the secondary structure with attenuated total reflection Fourier transform infrared spectroscopy, 100 µl of a 1 mg/ml solution of IAA-SC3 was dried on a germanium plate, and the spectra were recorded following the same procedure as described for native SC3 (5).

*Fluorescence Spectroscopy*—To test whether IAM-SC3 forms amyloid-like structures upon self-assembly, the increase in ThT fluorescence was determined with the procedure described previously (14). After trifluoroacetic acid treatment, IAM-SC3 was dissolved at 5 µg/ml in 50 mM phosphate buffer, pH 7.0, 3 µM ThT. The fluorescence at 482 nm (excitation = 450 nm) was followed for 3 h on a SPF-500C spectrofluorometer (SLM Aminco) operating with a 300 watt Xenon lamp type 300 UV. After three h the emission spectrum between 470 and 600 nm was determined.

*Binding to Teflon*—The coating of Teflon by SC3 and IAA-SC3 was assessed essentially as described by Wösten *et al.* (3). Thoroughly cleaned Teflon sheets were incubated for 16 h in 20 µg/ml $^{35}$S-labeled SC3 followed by three washes with water for 10 min each at pH 1.5 or 7.0. The amount of adsorbed $^{35}$S-labeled protein was determined by scintillation counting before and after hot SDS extraction and subsequent washes with water.

*Atomic Force Microscopy*—40 µl of 8 µg/ml IAA-SC3 or IAM-SC3 was applied to freshly cleaved mica disks (Metafix, Montdidier, France) mounted on metal disks coated with Teflon adhesive tape (BYTAC, Norton Performance Plastics Corp.) and allowed to dry. Images were acquired under ethanol with a Nanoscope IIIa multimode atomic force microscope (Digital Instruments, Santa Barbara, CA) equipped with a J (120 µm) scanner operated in a constant force contact mode. Oxide-sharpened silicon nitride tips mounted on cantilevers with a nominal force constant of 0.06 N/m, scanning rates of 8–15 Hz, and a scan angle
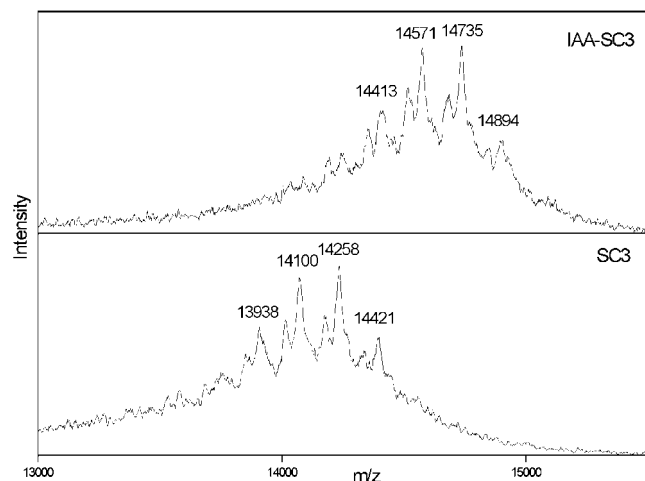
FIG. 3. **Matrix-assisted laser desorption-ionization mass spectrum of native SC3 (*lower panel*) and after reaction with iodoacetic acid (*upper panel*).** The heterogeneity in the glycosylation results in the appearance of peaks 162 daltons apart. Alternative splicing of a glycine at the amino terminus of the mature protein gives rise to a peak 57 daltons lower in mass. The mass difference between native SC3 and IAA-SC3 indicates that all eight cysteine residues have reacted and that there was no change in the degree of glycosylation. Reaction with iodoacetamide gave the same result (data not shown).

of 90° were used. The force was kept constant at the lowest possible value by continuously adjusting the set point during imaging. No processing was applied to the images other than flattening.

## RESULTS

*Carboxymethylation of SC3*—The CD spectrum of SC3 in the presence of DTT or guanidine could only be determined between 212 and 250 nm, because of absorption of light by DTT and guanidine in this region. Addition of 10 mM DTT or 5.4 M guanidine alone only slightly changed the CD spectrum of monomeric SC3, indicating only limited unfolding of the protein (Fig. 2). The spectrum changed to that of an unfolded protein in the presence of both 10 mM DTT and 5.4 M guanidine, as did the spectrum of IAA-SC3 (see "Structural Changes"). Under these conditions the disulfides are broken, and the free cysteines can be modified.

Carboxymethylation of SC3 was performed as described under "Experimental Procedures." Matrix-assisted laser desorption-ionization mass spectrometry (Fig. 3) showed the expected mass increase of 472 daltons after carboxymethylation of the eight cysteine residues with iodoacetic acid (IAA-SC3) or iodoacetamide (IAM-SC3). IAA-SC3 was readily soluble in water, probably because of the solubilizing effect of the negative charges. In contrast dialyzed and lyophilized SC3 and IAM-SC3 could only be solubilized by a trifluoroacetic acid treatment. After removing the trifluoroacetic acid and dissolving in water, IAM-SC3 spontaneously precipitated from solution, in contrast to native SC3, which remained soluble for days.

*Structural Changes*—Three different conformations of native SC3 have been identified (5). In water, SC3 is in the so-called monomeric conformation. Upon binding to a hydrophobic solid surface the protein is arrested in an intermediate α-helical state, whereas upon self-assembly at the air-water interface, rodlets are formed in a β-sheet conformation similar in structure to amyloid fibrils[1]. The secondary structure of IAA-SC3 and IAM-SC3 in water and after interaction with the air-water or Teflon-water interface was studied using circular dichroism and infrared spectroscopy. The CD spectrum of IAA-SC3 in water at pH 7.0 is clearly different from that of native SC3 and typical for a protein containing mainly random coil structure, as is apparent from the minimum at 200 nm (Fig. 4,
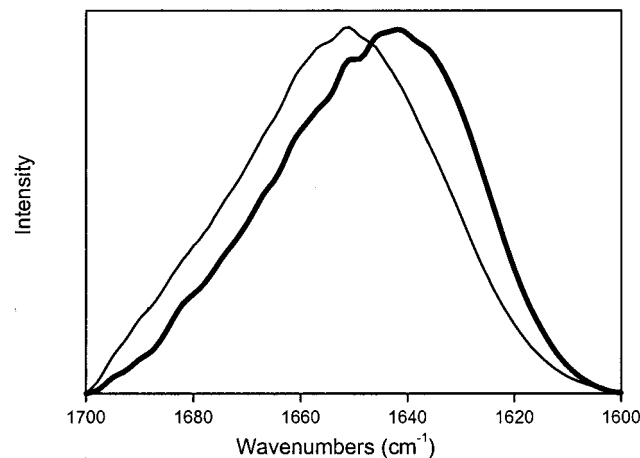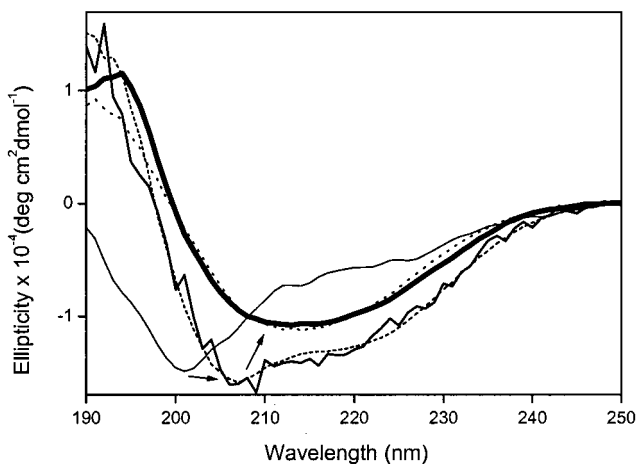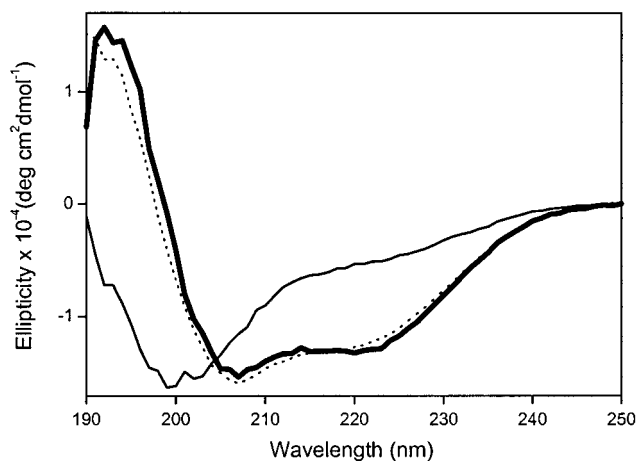






FIG. 4. **The secondary structure before and after assembly of IAA-SC3 and IAM-SC3 followed with circular dichroism spectroscopy.** The *top panel* shows the CD spectrum of IAA-SC3 in the monomeric form (*thin line*) and after assembly on colloidal Teflon (*thick line*). For comparison, the CD spectrum of native SC3 assembled on Teflon in the "α-helical form" is shown (*dotted line*). The *middle panel* shows the CD spectrum of IAM-SC3 immediately after dissolving the protein in buffer at 4 °C (*smooth thin line*) and after 5 (*jagged thin line*) or 90 min at 25 °C (*thick line*). For comparison, the CD spectra of native SC3 adsorbed to Teflon in the α-helical form (*dashed line*) or assembled at the air-water interface in the "β-sheet form" (*dotted line*) are shown. The *bottom panel* shows the attenuated total reflection-Fourier transform infrared spectra of IAA-SC3 dried on a germanium plate before (*thin line*) and after (*thick line*) deuteration.
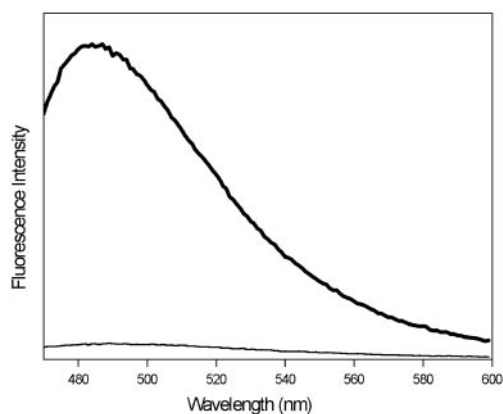
FIG. 6. **Binding of SC3 and IAA-SC3 to hydrophobic Teflon sheets before (*black*) and after (*gray*) extraction with hot SDS.** The numbers below the columns indicate the pH during incubation and washing with water (*black bars*) or during extraction with hot SDS (*gray bars*).

*top panel*). This was confirmed by attenuated total reflection Fourier transform infrared spectroscopy. A broad peak was observed with a maximum at 1652 cm$^{-1}$ that shifted to 1640 cm$^{-1}$ upon deuteration (Fig. 4, *bottom panel*). This peak is typical for random coil proteins (15). This shows that SC3 is unfolded after opening of the disulfide bridges and introduction of eight negative charges. The CD spectrum did not change upon rapid agitation of the sample, which causes native SC3 to self-assemble at the air-water interface in the amyloid-like $\beta$-sheet form (5). However, upon addition of colloidal Teflon, the IAA-SC3 rapidly folded into a conformation that exhibits the same CD spectrum as the $\alpha$-helical state of SC3; this is the same state that is found after interaction of native SC3 with Teflon (Fig. 4, *top panel*). When the colloidal particles were spun down, no IAA-SC3 could be detected in the supernatant, indicating that it was bound to the Teflon.

IAM-SC3 behaves quite differently from IAA-SC3. After disassembly of IAM-SC3 with trifluoroacetic acid, evaporation of the solvent, and dissolving the protein in water at 4 °C, IAM-SC3 showed the same CD spectrum as IAA-SC3 in water (Fig. 4, *middle panel*). However, the CD spectrum changed when the temperature was raised to 25 °C. After 5 min the CD spectrum shifted to that of the $\alpha$-helical state and in about 90 min it changed to a spectrum representative of $\beta$-sheet structure. The spectrum of IAM-SC3 in the $\alpha$-helical state is similar to that of native SC3 bound to Teflon, whereas the spectrum of IAM-SC3 in the $\beta$-sheet form is similar to that of the native protein at the air-water interface (Fig. 4, *middle panel*). The change in the spectrum of IAM-SC3 was accompanied by the formation of aggregates. The IAM-SC3, assembled in the $\beta$-sheet form, did not change its conformation upon addition of colloidal Teflon. However, when Teflon was added immediately after dissolving the protein, a CD spectrum was observed that was a combination of the so-called "$\alpha$-helical spectrum" and the "$\beta$-sheet spectrum" (data not shown). Thus before it precipitates IAM-SC3 can also be trapped in the $\alpha$-helical state on Teflon. In the case of IAA-SC3 the precipitation in the $\beta$-sheet form was also observed when the pH of an aqueous solution was lowered below 2.5 (data not shown). This shows that the negative charges attached to the cysteine residues prevent IAA-SC3 from aggregating.

The fluorescent dye ThT shows such a strongly increased fluorescence intensity when bound to amyloid fibrils that this characteristic has become a tool to detect the presence of amyloid-like protein fibrils. We have shown that native SC3 self-assembles into rodlets that resemble amyloid fibrils as demonstrated by an increase in ThT fluorescence[1] (14). The self-assembled IAM-SC3 also increased the ThT fluorescence (Fig.
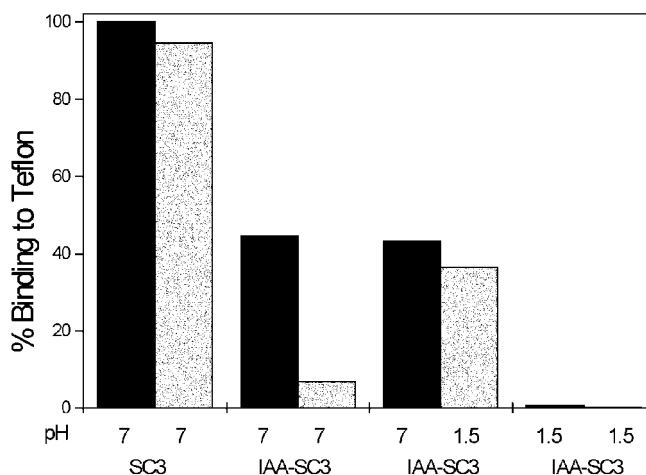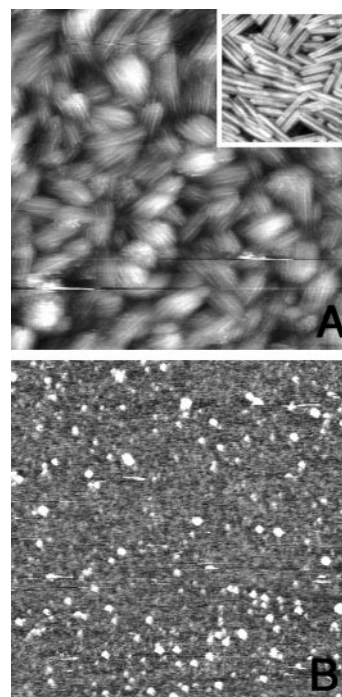


FIG. 7. *A*, IAA-SC3 rodlets are visible after drying the protein on the surface of mica (scan size (Z-scale), 2 $\mu$m (40 nm)). *A* shows a 250 $\times$ 250-nm image of IAA-SC3 rodlets (Z-scale, 15 nm). *Inset* shows a 100 $\times$ 100-nm image of the native SC3 rodlets (Z-scale, 30 nm). *B*, no rodlets were found when IAM-SC3 was dried on the surface of mica. Scan size (Z-scale), 2 $\mu$m (8 nm).

5), indicating that the IAM-SC3 precipitation is accompanied by formation of amyloid-like structures in the same way as native SC3.

*Binding to Teflon*—SC3 binds very strongly to hydrophobic surfaces such as Teflon (3). 1.5 mg of protein is enough to cover about 1 m$^2$ of the hydrophobic solid (5). Even heating for 10 min at 100 °C in 2% SDS only removes about 10% of the protein from the surface (3). We tested whether the folding of IAA-SC3 on Teflon, as shown by CD, also resulted in a strong binding. A Teflon sheet was incubated overnight in 30 $\mu$g/ml of $^{35}$S-labeled IAA-SC3 at pH 7.0. Only 0.7 mg/m$^2$ IAA-SC3 bound to Teflon compared with 1.5 mg/m$^2$ for native SC3 (Fig. 6). This is probably caused by electrostatic repulsion between the protein mol-

ecules during adsorption. When the Teflon sheet was extracted with hot SDS at pH 7.0, 85% of the protein was removed from the surface, indicating a weaker binding of IAA-SC3 to the Teflon surface compared with that of SC3. To test the effect of the charges on the binding, the sheets were extracted with SDS at pH 1.5. In this case, only 16% of the protein was removed from the sheet (Fig. 6), showing that the weak binding is indeed caused by the introduction of eight negative charges and not by a difference in conformation. When IAA-SC3 was incubated with the Teflon sheets at pH 1.5, no binding to Teflon was found, most likely because the protein precipitated before it could interact with the hydrophobic solid, as was found for IAM-SC3.

*Rodlet Structure*—When native SC3 is dried down and imaged with electron microscopy (6, 16)[2] or atomic force microscopy[2] (5), a typical rodlet pattern is observed with the same morphology as found on the surface of aerial hyphae (17). These structures represent the hydrophobic side of the SC3 film. Atomic force microscopy images (Fig. 7*A*) show that rodlet structures are also formed after drying down a solution of IAA-SC3. However, only aggregates were found for IAM-SC3 in the β-sheet form after a 90-min incubation in water (Fig. 7*B*), probably because the protein had self-assembled before the rodlets could be formed on the air-water interface.

### DISCUSSION

SC3 is a very stable protein. The CD spectrum of the monomeric form hardly changes in 10 mM DTT or in 5.4 M guanidine (Fig. 2). In addition, heating to 90 °C or changing the pH between 3 and 12 does not change the secondary structure of the protein (data not shown). SC3 only unfolds after reduction of the disulfide bridges with 10 mM DTT in 5.4 M guanidine. This shows that the disulfide bridges are essential for the stability of the monomeric form of SC3.

In general the conformation of proteins that adsorb on a hydrophobic solid support varies from a native or nearly native conformation to an extensively unfolded conformation (18, 19). The remarkable refolding of IAA-SC3 on Teflon, with eight additional negative charges, demonstrates the high propensity of the hydrophobin to refold on a hydrophobic surface and is a clear illustration of the specific interaction of hydrophobins with hydrophobic substrates. However, the stability of the IAA-SC3 layer is decreased compared with a layer of assembled native SC3. This instability is not caused by a difference in structure but by the negative charges at pH 7.0, as shown by the recovery of the strong binding when the extraction with SDS was performed at low pH. Furthermore, the IAA-SC3 is still capable of forming rodlets (Fig. 7*A*). This confirms the observation that intermolecular disulfide bridges are not involved in the self-assembly process.

All hydrophobins have the same distribution of cysteine residues, and they probably have the same pattern of disulfide bridges. They are produced as monomeric proteins and can self-assemble into more or less insoluble films after interaction with a hydrophobic-hydrophilic interface. For SC3 it has been shown that this film consists of amyloid fibrils[1]. For many amyloid proteins the polymerization is initiated by a partially folded state of the protein (20, 21). Therefore it is understandable that opening of the disulfide bridges (which destabilizes the monomeric form) increases the propensity of hydrophobins to form amyloid fibrils.

IAM-SC3 in water spontaneously self-assembles in an amyloid-like β-sheet form in the absence of a hydrophobic-hydrophilic interface, but it first goes through a short-lived α-helical state. This corroborates the observation that the α-helical form also in native SC3 is an intermediate in the self-assembly process[2]. We propose that the disulfide bridges stabilize the monomeric form of hydrophobins and prevent premature self-assembly (before interaction with a hydrophobic-hydrophilic interface), which would result in protein aggregates within the cell or its aqueous environment. The eight conserved cysteine residues in hydrophobins are thus essential for the function of hydrophobins at hydrophobic-hydrophilic interfaces during fungal development.

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