# Structural Characterization of the Hydrophobin SC3, as a Monomer and after Self-Assembly at Hydrophobic/Hydrophilic Interfaces

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ABSTRACT Hydrophobins are small fungal proteins that self-assemble at hydrophilic/hydrophobic interfaces into amphipathic membranes that, in the case of Class I hydrophobins, can be disassembled only by treatment with agents like pure trifluoroacetic acid. Here we characterize, by spectroscopic techniques, the structural changes that occur upon assembly at an air/water interface and upon assembly on a hydrophobic solid surface, and the influence of deglycosylation on these events. We determined that the hydrophobin SC3 from *Schizophyllum commune* contains 16–22 O-linked mannose residues, probably attached to the N-terminal part of the peptide chain. Scanning force microscopy revealed that SC3 adsorbs specifically to a hydrophobic surface and cannot be removed by heating at 100°C in 2% sodium dodecyl sulfate. Attenuated total reflection Fourier transform infrared spectroscopy and circular dichroism spectroscopy revealed that the monomeric, water-soluble form of the protein is rich in $\beta$-sheet structure and that the amount of $\beta$-sheet is increased after self-assembly on a water-air interface. $\alpha$-Helix is induced specifically upon assembly of the protein on a hydrophobic solid. We propose a model for the formation of rodlets, which may be induced by dehydration and a conformational change of the glycosylated part of the protein, resulting in the formation of an amphipathic $\alpha$-helix that forms an anchor for binding to a substrate. The assembly in the $\beta$-sheet form seems to be involved in lowering of the surface tension, a potential function of hydrophobins.

## INTRODUCTION

Hydrophobins are small proteins, containing eight conserved cysteine residues (see Fig. 1), that are widespread among fungi (Wessels, 1994, 1996, 1997). They form a more or less insoluble amphipathic membrane at hydrophobic/hydrophilic interfaces and are among the most surface-active biosurfactants known (Wösten and Wessels, 1997). In so-called Class I hydrophobins, this amphipathic membrane is highly insoluble and is characterized, on the hydrophobic side, by a typical rodlet pattern, which is also found on the outside of many aerial fungal structures (Wessels, 1997). SC3, a glycosylated hydrophobin from *Schizophyllum commune* (Ásgeirsdóttir, 1994), is the best studied hydrophobin to date. Disruption of the *SC3* gene affects the formation of aerial hyphae and the adherence of hyphae to hydrophobic surfaces (Wösten et al., 1994b; van Wetter et al., 1996). Intermolecular disulfide bridging does not seem to be important for the formation of rodlet structures during the assembly of SC3 (de Vries et al., 1993; Wösten et al., 1993, 1994b). It self-assembles on water-air, water-oil, and water-hydrophobic solid interfaces into an amphipathic film that cannot be disassembled by heating at 100°C for 10 min in a solution of 2% sodium dodecyl sulfate (SDS). The monomeric state of the protein can only be reestablished by dissolving the protein in pure trifluoroacetic acid (TFA) or formic acid (de Vries et al., 1993; Wösten et al., 1993). Van der Vegt et al. (1996) have proposed that the protein layer, during assembly, extensively changes its conformation upon adsorption. In this study we focus on conformational changes that have been suggested to play an important role during the self-assembly of hydrophobins (Wessels, 1997) and on the role of glycosylation in these processes.

## MATERIALS AND METHODS

### Protein

SC3 was purified from the culture medium of strain 4-40 of *Schizophyllum commune* (CBS 340.81) as described previously (Wösten et al., 1993; Wessels, 1997). Before use the SC3 was disassembled with pure TFA and dried in a stream of nitrogen. The monomeric protein was dissolved in water or the desired buffer.

### Deglycosylation of SC3 and mass analysis

To analyze the carbohydrate attached to SC3, 1 mg of the protein was hydrolyzed in 0.5 ml 2 M TFA under nitrogen at 100°C for 3 h in a sealed vial. The precipitate formed was removed by centrifugation. The supernatant was lyophilized and taken up in 50 $\mu$l 10% *n*-propanol, and 10-$\mu$l samples were subjected to ascending thin-layer chromatography on silica or cellulose plates in *n*-propanol/water/ethylacetate (7:2:1) at 55°C. Silica plates were sprayed with sulfuric acid/methanol (1:1) followed by charring to detect organic compounds. Cellulose plates were sprayed with ninhydrin (0.2% in acetone) and left overnight at room temperature to detect amino compounds and subsequently sprayed with anilinephtalic acid in butanol

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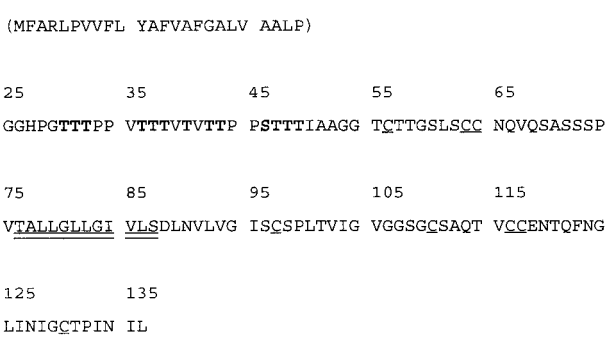


FIGURE 1 Amino acid sequence of SC3 (accession number P16933). The sequence is corrected compared to the previously published sequence (Schuren and Wessels, 1990), because it was found that the part of one intron contained the coding sequence for 12 amino acids. Edman degradation of the secreted protein revealed that the N-terminal 24 residues are split off as a signal sequence (Wessels et al., 1991). The cysteine residues are underlined; the residues that are predicted to be glycosylated are printed in bold, and the residues possibly involved in the $\alpha$-helix formation during assembly at the water-hydrophobic solid interface are double underlined (see Discussion).

followed by heating at 105°C to detect reducing sugars. The monosaccharides were converted to trimethylsilylated methyl sugars by incubating for 24 h in 2.0 M methanolic HCl at 85°C and analyzed after methanolysis by gas liquid chromatography (Kamerling and Vliegenthart, 1989), using a Varian 3600 gas chromatograph equipped with a J&W DB-1 capillary column. Parallel analysis of a derivatized monosaccharide standard was performed for identification and quantification. The carbohydrate content was also determined with the Anthrone reagent (Fairbairn, 1953), with mannose as a standard.

Deglycosylation of SC3 was achieved with trifluoromethanesulfonic acid (TFMSA) (Edge et al., 1981). A mixture of 0.5 ml TFMSA and 0.25 ml anisole was cooled on ice and added to 5 mg of lyophilized protein. After incubation for 3 h under $N_2$ on ice, the reaction was terminated by cautious addition of 1.5 ml 50% (v/v) aqueous pyridine and extracted three times with diethyl ether. The aqueous phase was dialyzed exhaustively against water and lyophilized.

MALDI-TOF mass spectrometry was used to analyze the glycosylation of SC3 samples. One microliter of a solution of SC3 ($\sim$100 $\mu$g/ml) was dried on a target, on top of which a 1-$\mu$l matrix of 20 mg/ml sinapinic acid in 40/60 (v/v) acetonitrile/0.1% TFA in water was dried. The spectra were recorded on a TofSpec E & SE Micromass mass spectrometer.

## Physical properties of interfaces

The hydrophobicity of the surfaces used for scanning force microscopy (SFM), attenuated total reflection–Fourier transform infrared (ATR-FTIR) spectroscopy, and coating experiments was assessed by water contact angle measurements, using the sessile drop technique (van der Mei et al., 1991). The surface tension was determined by axisymmetrical drop-shape analysis by profile (ADSA-P) on a 100-$\mu$l protein solution, as described by Noordmans and Busscher (1991).

The coating of Teflon by deglycosylated SC3 was assessed essentially following the procedure described by Wösten et al. (1994b), using deglycosylated $^{35}$S-labeled SC3. A thoroughly cleaned Teflon sheet was incubated for 16 h in 2 $\mu$g/ml deglycosylated SC3, followed by three washes with water for 10 min each. Half of the sheet was extracted for 10 min at 100°C in 2% SDS, again followed by three washes with water. The amount of adsorbed $^{35}$S-labeled SC3 was determined directly by counting the sheets in 1.5 ml scintillation liquid (Opti-gold; Hewlett Packard Instruments).

## Scanning force microscopy

A chemically patterned substrate was created with Micro-Contact Printing (Kumar and Whitesides, 1993; López et al., 1993; Thoughton et al., 1988) on 1 $\times$ 1 cm$^2$ Si-wafers on which 2 nm chromium and 100 nm gold were evaporated, following the same procedure as described by van der Vegte and Hadziioannou (1997). Stamps were created from photolithography patterns (masters) exhibiting 20 $\times$ 20 $\mu$m$^2$ squares separated by 20 $\mu$m. Lateral force mode and topographic mode images of the patterned substrate were recorded with a Topometrix Discoverer (TMX2010) atomic force microscope equipped with a closed liquid cell. All measurements were performed in ethanol (van der Vegte and Hadziioannou, 1997).

For imaging of the rodlet layer, a 100 $\mu$g/ml solution of SC3 in 40% ethanol in water was dried on a atomically flat bare mica substrate, the same procedure as used for electron microscopy (Wösten et al., 1993, 1994b). The surface was scanned with an unmodified $Si_3N_4$ tip in ethanol with a very low loading force to prevent deformation of the protein layer.

## ATR-FTIR spectroscopy

A Perkin Elmer 1720$\times$ Fourier transform infrared (FTIR) spectrophotometer equipped with a liquid nitrogen-cooled mercury-cadmium-telluride detector was used to collect the ATR-FTIR spectra. The samples were deposited on a germanium plate (50 $\times$ 20 $\times$ 2 mm; Harrick EJ2121). The spectrophotometer was purged with dry air for 30 min, and the sample was deuterated by flushing with $N_2$ saturated with $D_2O$ for 2 h. Good signal-to-noise was obtained for samples dried down from solution and then deuterated by averaging 128 scans at a resolution of 4 cm$^{-1}$. However, the lower signal-to-noise from samples consisting of only a 7–8-nm layer of protein after adsorption from solution required averaging of 512 scans at a resolution of 0.5 cm$^{-1}$. The secondary structure was determined from the amide I′ band, following the procedure described by Goormaghtigh et al. (1990, 1994). The monomeric nature of the protein dried down from 100 $\mu$l of a 1 mg/ml solution was demonstrated as follows. The dried protein was extracted in 2% SDS. More than 95% ran at the normal monomeric mass in SDS-PAGE, whereas no pellet was formed after centrifugation of the protein, indicating that the protein was almost completely in the monomeric form. Traces of TFA were removed by incubating the plate overnight under 100% ethanol in which the protein is not soluble (Wösten et al., 1993).

## Circular dichroism measurements

The circular dichroism (CD) spectra were recorded over the wavelength region 190–250 nm on an Aviv 62A DS CD spectrometer, using a 5-mm quartz cuvette. The temperature was kept constant at 25°C, and the sample compartment was continuously flushed with $N_2$. Spectra are the average of 10 scans, using a bandwidth of 1 nm, a stepwidth of 1 nm, and a 5-s averaging per point. The spectra were corrected by using a reference solution without the protein. Typically, a protein concentration of 3.5 $\mu$M in 20 mM phosphate buffer at pH 7.0 was used. For spectra of the monomeric protein, the protein was simply dissolved in buffer. For spectra of the protein assembled at the air/water interface, assembly was achieved by vigorously shaking the solution on a vortex or heating the solution to 95°C. Both methods gave the same CD spectrum. The exact protein concentration was determined with total amino acid analysis (Schuster, 1988), from which the mean residue molar ellipticity could be calculated. The spectra were not smoothed.

## Adsorption of SC3 on colloidal Teflon

For determination of the structure of the protein adsorbed on a hydrophobic surface, perfluoroalkoxy fluorocarbon Teflon was used (kindly donated by Dr. D. Levy, Du Pont de Nemours, Grand-Saconnex, Switzerland) (Maste et al., 1996). The baseline of the CD spectrum was flat for a solution of colloidal Teflon and could be measured up to 190 nm. Before use the

Teflon latex (type PFA 350) was filtered through glass wool. The average diameter of the Teflon spheres was determined by electron microscopy to be 129 nm. An adsorption isotherm was first determined to control the coverage. Varying concentrations of protein solution were incubated overnight in glass tubes with 0.98 mg Teflon (= 0.023 $m^2$). The Teflon and nonadsorbed protein were separated by centrifugation. The residual protein concentration in the supernatant was determined from the ellipticity in the CD at 209 nm. The adsorbed amounts were calculated from the mass balance. SC3 adsorbs on Teflon with a high-affinity adsorption isotherm and a well-defined plateau value of 1.5 $mg/m^2$. CD measurements of adsorbed protein were done with 28 $\mu$g SC3 in the cuvette and 0.21 $m^2$ of Teflon, at 9% surface coverage. This is a concentration in the high-affinity region of the adsorption isotherm, and all of the protein is adsorbed.

## RESULTS

### (De-)glycosylation of SC3

Hydrolysis of SC3 for 3 h in 2 M TFA at 100°C and analysis by thin-layer chromatography, and gas liquid chromatography showed that the protein contains 20 mannose residues. This result was confirmed by an Anthrone reaction, which indicated that 20–23 sugar residues are present. Thirteen different forms of SC3 were detected by MALDI-TOF mass spectrometry (Fig. 2, *top*). The theoretical average mass of nonglycosylated SC3 with intact disulfide bonds is 10,856 (de Vries et al., 1993). The mass spectrum indicates SC3 with 17–22 mannose residues (162 Da/residue). Edman degradation revealed that, in a fraction of the molecules, the N-terminal glycine was absent (Fig. 1) (Wessels et al., 1991), resulting in extra mass peaks 57 mass units lower than the mass of the complete sequence. All of the mannose residues were removed after incubation of SC3 in trifluoromethanesulfonic acid (TFMSA) for 3 h (Fig. 2, *middle*).

### Comparison of the in vivo composition and secondary structure with that of purified SC3

SC3 is secreted by *S. commune* into the growth medium in a water-soluble, monomeric form and subsequently assembles into insoluble films at an air/water interface. During purification, the assembled form of SC3 is collected and then treated with 100% TFA to dissociate it into water-soluble monomers (Wösten et al., 1993). After this treatment, the protein is still fully able to reassemble and form rodlet-like structures. To check whether the secondary structure of monomeric SC3 is changed during disassembly in TFA, the CD spectrum of the purified protein was compared to that of the growth medium after 5 days of growth of *S. commune* (Fig. 3) at 25°C. This spectrum (*thin line*) and that of purified SC3 (*thick line*) are nearly identical, suggesting that the secondary structures of the purified and growth medium forms of SC3 are the same. The only significant difference between the corrected medium spectrum and that of purified SC3 is seen in the low-wavelength region. It can be attributed to differences in nondialyzable metabolic end-products of *S. commune* with and without SC3.

The MALDI-TOF mass spectra of the same dialyzed medium are shown in Fig. 2 (*bottom*). There are only a few components visible in this unpurified medium, one of which

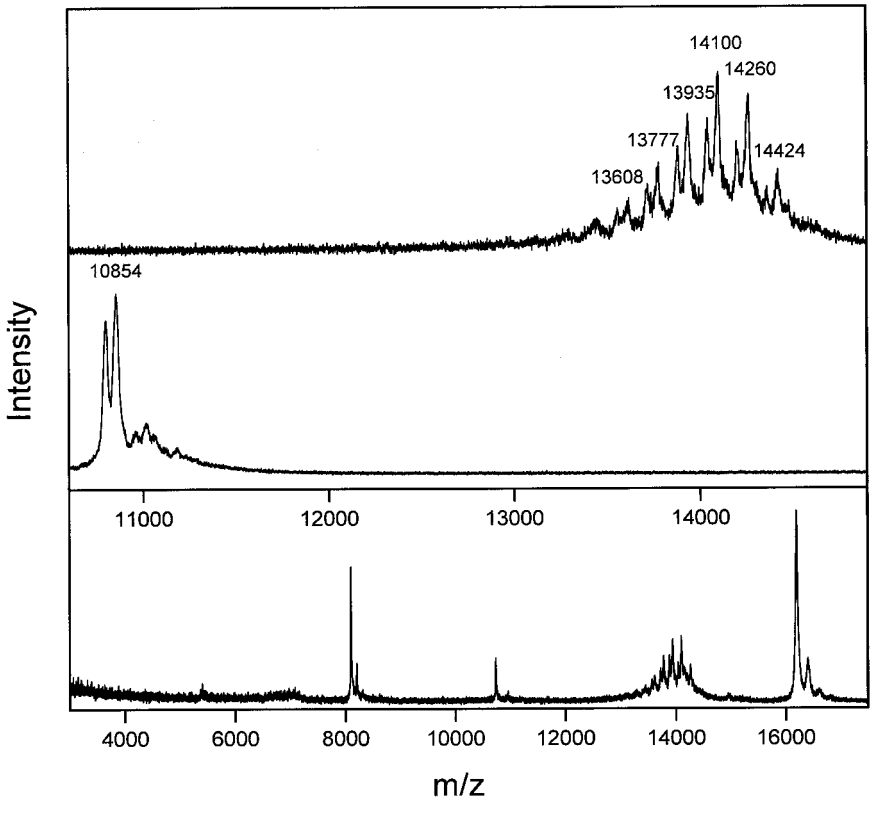


FIGURE 2 MALDI-TOF mass spectrum of purified SC3 (*upper panel*) and purified SC3 after deglycosylation with TFMSA (*middle panel*). The lower panel is the mass spectrum of the dialyzed growth-medium from a 5-day-old culture of *S. commune*. The glycosylated SC3 is clearly visible at 14000 *M/Z*, in addition to several other protein components of various *M/Z*. See Materials and Methods and text for details.

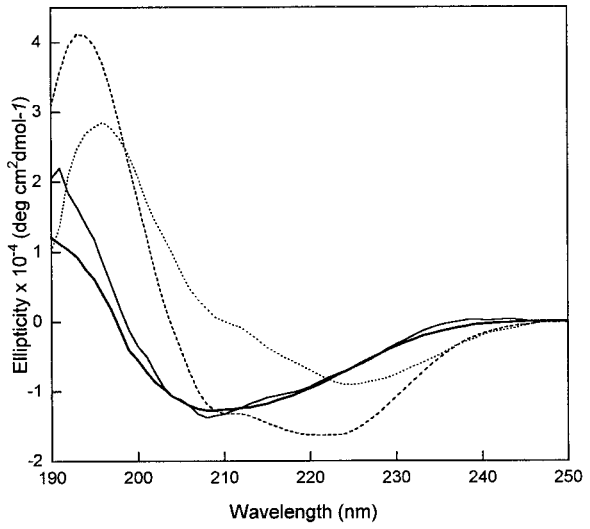


FIGURE 3 Comparison of CD spectrum of isolated SC3 (*thick line*) and the CD spectrum of SC3 in the medium of *S. commune* (*thin line*). *S. commune* (strain 4–40) and a strain of *S. commune* containing a disruption of the *SC3* gene, but otherwise genetically identical (Wösten et al., 1994b; van Wetter et al., 1996), were grown on minimal medium in a 9-cm polystyrene petri dish. After 5 days the medium was separated from the mycelium and extensively dialyzed against three changes of water, using a membrane with a molecular weight cut-off of 3500 Da. The CD spectrum of the medium of the wild-type fungus (*dashed line*) and the medium of the SC3 disruptant strain (*dotted line*) were recorded without any further purification. The spectra were standardized by correcting for the volume of the media from which the samples were taken. The difference between these two spectra is plotted as a thin line. The CD spectra of the media were smoothed.

is SC3, centered at 14,000 *M/Z*. It shows the same mass distribution and glycosylation pattern as the purified protein. Consequently, no mannose residues are removed during the treatment with 100% TFA, and no secondary structural changes result from this treatment.

## Interfacial properties of deglycosylated SC3

Wösten et al. (1994b) showed that the water contact angle of a Teflon surface decreased from 108° to 48° after incubation for 16 h in a solution of SC3. Heating of the surface at 100°C for 10 min in 2% SDS removed only 13% of the protein and resulted in a contact angle of 62°. With deglycosylated SC3, the contact angle decreased from 108° to 66 ± 7°. Thus there is a correlation between the presence of mannose residues and the value of the contact angle of Teflon coated with SC3. Heating the surface for 10 min at 100°C in 2% SDS removed 72% of the deglycosylated protein, resulting in a contact angle of 94 ± 2°. Apparently the removal of the hydrophilic mannose residues from the protein also results in weaker binding of SC3 to the hydrophobic surface. The surface activity of SC3, however, seems not to be affected by deglycosylation; the surface tension of water decreases from 72 $mJ/m^2$ to 32 $mJ/m^2$ after assembly of deglycosylated SC3 at an water-air interface as measured with ADSA-P. The same decrease was measured with glycosylated SC3 (van der Vegt et al., 1996).

## Scanning force microscopy with chemical sensitivity

Wösten et al. (1994b, 1995) have reported a correlation between the adsorption of SC3 and the hydrophobicity of the sorbent; adsorption on a hydrophobic surface resulted in a hydrophilic protein-coated surface, whereas adsorption on a hydrophilic surface resulted in a hydrophobic protein-coated surface. Scanning force microscopy (SFM) can be used to visualize these chemical differences because it is possible to introduce chemical sensitivity by chemically modifying the SFM probe (Frisbie et al., 1994; Noy et al., 1995; Green et al., 1995; Thomas et al., 1995; van der Vegte and Hadziioannou, 1997). A chemical pattern of 20 × 20 $\mu$m squares of hydrophobic methyl groups was created in an environment of hydrophilic carboxylate groups by microcontact printing. The contact angle was determined to be 110° on the hydrophobic surface and less than 10° on the hydrophilic surface, as described in Materials and Methods. Tips functionalized with amide groups capable of hydrogen bonding show the highest friction (Fig. 4 *A*, *light areas*) on the hydrophilic carboxylate areas in the lateral force image mode, whereas the hydrophobic methyl areas show low friction and appear as dark areas. In the topographic imaging mode (Fig. 4 *B*), no height differences are detected between areas functionalized with methyl or carboxylate groups, because alkane thiols of the same length were chosen. SC3 and the reference protein, bovine serum albumin (BSA), were adsorbed on this patterned surface overnight from a 10 $\mu$g/ml solution at pH 7.0. The lateral force image showed no contrast between the hydrophobic and hydrophilic areas, indicating adsorption of proteins in both areas. The surface was then heated for 10 min at 100°C in a solution of 2% SDS. In the control experiment with BSA, the lateral and the topographic force image indicated complete removal of the protein layer from the entire surface by this treatment (data not shown). However, SC3 remained strongly bound to the hydrophobic squares in a 7–8-nm-thick layer, whereas it was completely removed from the surrounding hydrophilic carboxylate groups (Fig. 4 *D*). The lateral force image (Fig. 4 *C*) shows the reverse contrast in comparison to Fig. 4 *A*; there is a higher interaction between the hydrophilic tip and the protein film than between the tip and the surrounding carboxylate-covered surface. This confirms that SC3 adsorbed to a hydrophobic surface results in a 7–8-nm-thick protein film whose exposed surface is hydrophilic.

## Imaging of the rodlet layer

Rodlet structures composed of assembled hydrophobin are found on the outside of aerial hyphae of *Schizophyllum commune* and on the surface of many fungal spores. A

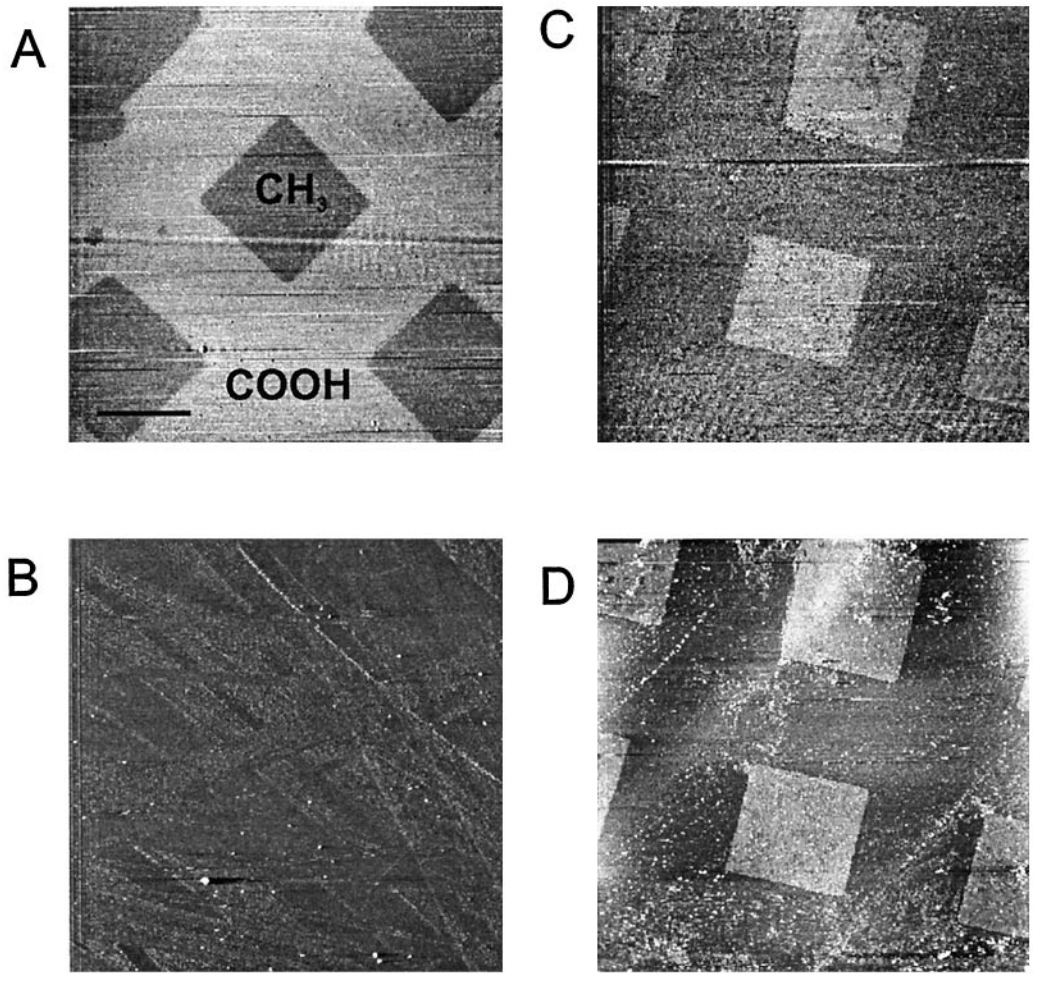


FIGURE 4 Scanning force microscopy images in the lateral force mode, indicating the interaction between the tip and the surface (*A* and *C*) and topography mode, indicating the height of the sample (*B* and *D*). (*A* and *B*) The clean, chemically patterned surface of hydrophobic methyl 20 × 20 $\mu m^2$ squares in an environment of hydrophilic carboxylate groups. (*C* and *D*) The surface after overnight incubation in a solution of 10 $\mu$g/ml SC3 at pH 7, followed by heating the surface in 2% SDS for 10 min at 100°C. The lateral force mode measurements were performed with a Topometrix Discoverer (TMX2010) atomic force microscope with a closed liquid cell. All measurements were performed in ethanol. High lateral force and raised topological surfaces are indicated by the lighter color. The bar represents 20 $\mu$m.

similar rodlet pattern is found when SC3 is dried on a surface. In this case the hydrophobic side of the protein film is exposed and can be studied by electron microscopy (Wösten et al., 1993, 1994b). We repeated this experiment on a mica surface and imaged the surface with a scanning force microscope. Although we also found rodlet structures after drying an aqueous solution of SC3, we found that the highest resolution could be obtained after drying down SC3 from a solution containing 40% ethanol. The same pattern of parallel rods was found with an average diameter of 9–15 nm (Fig. 5).

## ATR-FTIR spectroscopy

The shape and frequency of the amide I′ band assigned to the $\nu$(C═O) of the peptide bond is sensitive to the secondary structure of proteins (Goormaghtigh et al., 1994). Because we are concerned with structural changes that occur upon film formation, we used ATR spectroscopy to study SC3 before and after assembly on a specially prepared germanium plate. The ATR-FTIR spectrum of monomeric SC3 was recorded after drying down 100 $\mu$l 1 mg/ml of the protein on the plate. After this procedure at least 95% of the protein is in the monomeric state (see Materials and Methods). After deuteration, the spectrum shows one major band at 1636 $cm^{-1}$ (*thick line* in Fig. 6). The ATR-FTIR spectrum of SC3 assembled on a water-air interface was recorded after deuteration (*dotted line*, Fig. 6). The ATR-FTIR spectrum of SC3 assembled on a hydrophobic surface (Fig. 6, *thin line*) was recorded by using a germanium plate that had been silanized to make it hydrophobic, according to the procedure described by Baty et al. (1996). The contact angle of the silanized plate was more than 100°. Only a 7–8-nm layer of protein is measured in this case (*thin line*, Fig. 6), and therefore the intensity of the amide I′ band is much lower than in the previous spectra. For a better com-

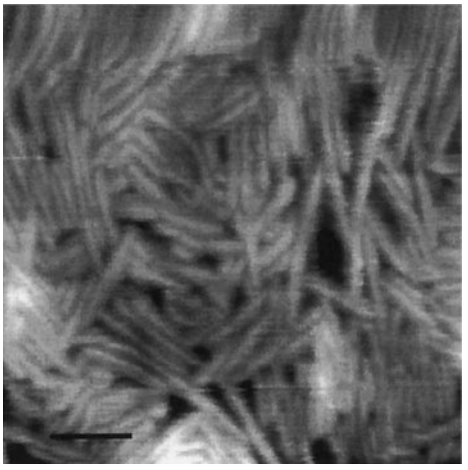

FIGURE 5 Scanning force microscopy pictures of the rodlet pattern after drying down 100 $\mu$g/ml SC3 in water on a mica surface. The measurement was performed in ethanol. The bar represents 100 nm.

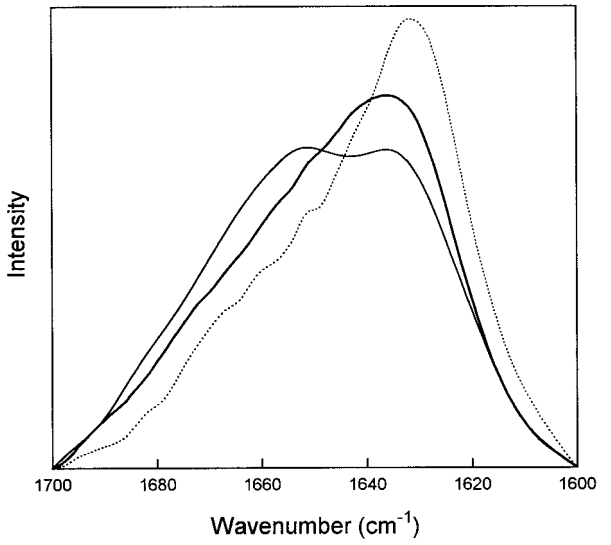


FIGURE 6 Attenuated total reflection–Fourier transform infrared spectra of SC3 supplied on a germanium plate. SC3 was deposited on the plate in a monomeric form (*thick line*), after assembling SC3 at the water-air interface by vortexing 100 $\mu$l 1 mg/ml protein for 3 min at high speed (Wösten et al., 1993). The assembled protein was spun down for 10 min in a table centrifuge. The supernatant was removed and the pellet was resuspended in 100 $\mu$l water. The suspension was dried on a germanium plate and the spectrum was recorded (*dotted line*); SC3 was assembled, overnight, on a hydrophobic (silanized) germanium plate and subsequently heated at 100°C for 10 min in 2% SDS, following the same procedure as used for the SFM (*thin line*). The vertical scale is arbitrary.

parison, all of the spectra were adjusted to the same peak area. When this experiment was repeated with deglycosylated SC3, nearly all of the protein was removed during the heating in SDS, and only a very low signal could be detected, just above the noise, which was similar in shape to the spectrum from the protein assembled at the air-water interface (data not shown). Deglycosylation, as shown above, results in a lower association strength on hydrophobic surfaces and a different secondary structure. The secondary structure of the monomeric and assembled protein was estimated by Fourier self-deconvolution and curve fitting of the amide I′ region (Goormaghtigh et al., 1994) (Table 1). Unfortunately, the 7–8-nm-thick protein layer after adsorption on hydrophobic germanium yields a spectrum of too low an intensity to accurately determine the secondary structure content. The increase in $\alpha$-helix content, however, is evident.

### Circular dichroism measurements of (deglycosylated) SC3

The CD spectrum of monomeric SC3 (Fig. 7, *thick line*) changes upon assembly on the water-air interface (*dotted line*). The spectrum after assembly is characteristic for a protein containing mainly $\beta$-sheet structure. When an excess of colloidal Teflon was added to a solution of SC3 to provide a hydrophobic surface for assembly (coverage 9%), the spectrum changes within a few seconds, resulting in a spectrum with minima at 205 nm and at 218 nm (*thin line*), indicative of the formation of $\alpha$-helix (Chang et al., 1978). The band at 205 nm is also seen when SC3 is dissolved in 25% trifluoroethanol (TFE) (*dashed line*). After deglycosylation, the CD spectrum of monomeric SC3 changes (data not shown); it has the same shape as the CD spectrum of normal glycosylated SC3 in 25% TFE, but the spectrum hardly changes when the protein is dissolved in 25% TFE. This indicates that the glycosylated part of the protein is sensitive toward the polarity of the environment. After assembly at the water-air interface by vigorous shaking, the spectrum changes toward the spectrum of a $\beta$-sheet-like protein in the same way as glycosylated SC3. Assembly on the water-hydrophobic solid interface also shows a change of the CD spectrum; however, the induction of $\alpha$-helix is not as pronounced as for the glycosylated SC3.

## DISCUSSION

MALDI-TOF mass spectrometry and CD spectra of purified SC3 and the growth medium of the fungus clearly show that the TFA treatment during the purification changes neither the degree of glycosylation nor the secondary structure composition of the protein. Purified SC3 can still self-assemble into a rodlet layer, showing that the purified

**TABLE 1 Secondary structure of SC3 dried down on a germanium plate, as calculated from deconvolution and curve fitting analysis of the amide I′ recorded by ATR-FTIR spectroscopy**

| | $\alpha$-Helix (%) | $\beta$-Sheet (%) | $\beta$-Turn (%) | Random coil (%) |
|---|---|---|---|---|
| Monomeric | 23 | 41 | 16 | 20 |
| Self-assembled on water-air interface | 16 | 65 | 9 | 10 |

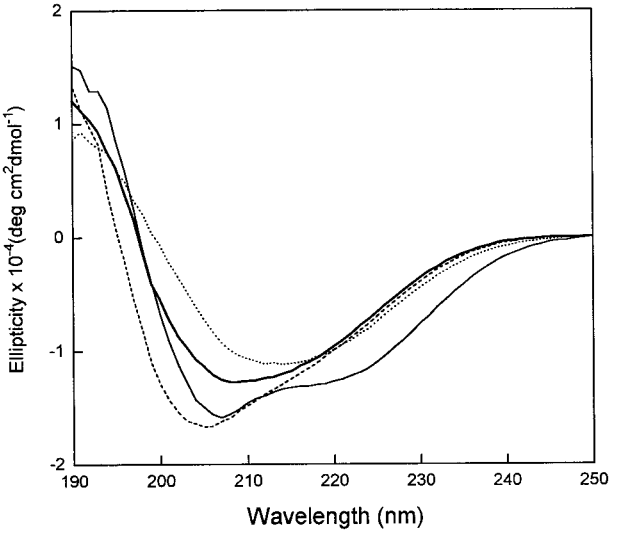


FIGURE 7 Unsmoothed circular dichroism spectra of SC3. SC3 in the monomeric form (*thick line*), SC3 after assembly at the water-air interface by shaking (*dotted line*), assembled SC3 dissolved in 25% TFE (*dashed line*), and SC3 assembled on a hydrophobic colloidal Teflon surface (*thin line*). Spectra are the average of 10 scans corrected by using a reference solution without the protein. Typically, a protein concentration of 3.5 $\mu$M in 20 mM phosphate buffer at pH 7.0 was used.

protein retains properties that it possessed in the native state in the fungus.

## Attachment of SC3 to surfaces

Chemically patterned self-assembled monolayers combined with SFM can be used to study the interaction of (bio-) molecules with specific chemical groups on the surface. SC3 adsorbs strongly to hydrophobic surfaces, as does BSA (Lee and Ruckenstein, 1988). Topographic measurements show that after heating in 2% SDS, a SC3 layer of 7–8 nm remains bound to the hydrophobic surface. The height of this layer may be underestimated because of pressure exerted on the protein layer by the applied load on the tip during scanning. The lateral force during scanning with the hydrophilic tip can give information about the nature of the protein film. The strong interaction between the hydrophilic tip and the protein layer (Fig. 4 *C*) can be explained both by the softness of the protein film, which creates a higher contact area between the tip and the protein surface, and by the hydrophilic nature of the surface after binding of SC3 to a hydrophobic substrate, as shown earlier by contact angle measurements (Wösten et al., 1994b, 1995). We could not obtain a high-resolution image of the hydrophilic side of the protein film, probably because the protein film has a soft surface.

## (De-)glycosylation of SC3

The conserved feature of all hydrophobins is the spacing of the eight cysteine residues. They appear in two regions of -C-$X_{5-9}$-C-C-$X_{6-39}$-C- (Fig. 1). There are three observations that indicate that the N-terminal 32 amino acids before the first cysteine are the part of the sequence of SC3 that is glycosylated. First, the threonines were not detected during N-terminal sequencing, probably because they have attached sugar residues. Second, a prediction program for O-glycosylation of eukaryotic proteins (Hansen et al., 1997a, b) predicts that only the threonines in this part of the sequence and only one serine (residue 46) are likely to be glycosylated (Fig. 1). Third, SC3 and POH2 from *Pleurotus ostreatus* (unpublished result, Ásgeirsdóttir), the only hydrophobins known to be glycosylated, have a longer N-terminal stretch before the first cysteine residue than most other hydrophobins. For example, the hydrophobin ABH1, from *Agaricus bisporus*, has properties similar to those of SC3, but is not glycosylated and possesses a much shorter N-terminal section (Lugones et al., 1996). The glycosylation might be important for hydrophobins with a long stretch before the first cysteine, helping to keep this domain in a hydrophilic environment. In contrast to the CD spectrum of glycosylated SC3, the CD spectra of deglycosylated SC3 in water and in 25% TFE are very similar, indicating a similar secondary structure. Assuming that, indeed, the mannose residues are present in the N-terminal region, these observations suggest that the conformation of the N-terminal part of the glycosylated protein is sensitive to the polarity of the environment.

The contact angle of Teflon coated with deglycosylated SC3 was 66°, higher than the 48° found for glycosylated SC3 (Wösten et al., 1994b, 1995). A study of the atomic composition of this layer by x-ray photoelectron spectroscopy suggests that the mannose residues are present on the hydrophilic side of the layer (Wösten et al., 1994b), which explains the higher contact angle obtained with deglycosylated SC3.

## Structure of monomeric SC3 and after assembly on the water-air interface

The secondary structure of the protein was predicted by profile fed neural network systems from Heidelberg (Rost and Sander, 1993, 1994; Rost et al., 1994). The cysteine-rich region was predicted to consist of alternating $\beta$-sheet and loop structure, whereas the N-terminal 32 amino acid segment before the first cysteine residue, which probably contains the mannose residues, was predicted to consist entirely of loop structures (Fig. 8). ATR-FTIR spectroscopy, CD spectroscopy, and secondary structure prediction all indicate that all forms of SC3 consist mainly of $\beta$-sheet structure. Furthermore, the CD and ATR-FTIR spectra suggest that more $\beta$-sheet structure is formed upon assembly on the water-air interface. Neither the conformational changes upon assembly at the water-air interface of SC3, nor the surface tension of water seem to be influenced by the deglycosylation of the protein. This suggests that the non-glycosylated part of the protein, probably the region con-

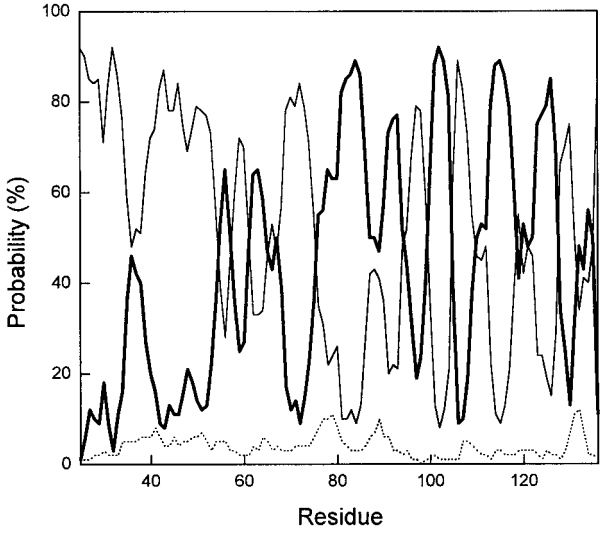


FIGURE 8 Probability for assigning $\alpha$-helix (*dotted line*), $\beta$-sheet (*thick line*), or loop structure (*thin line*) in the peptide chain of SC3, predicted by profile fed neural network systems from Heidelberg.

taining the disulfides, is important for the increase in $\beta$-sheet content upon assembly at the water-air interface.

## Structure of SC3 after assembly on a hydrophobic substrate

The driving force for the strong interactions between hydrophobins and hydrophobic surfaces is unclear. CD and ATR-FTIR spectra suggest that $\alpha$-helix is formed upon assembly on a hydrophobic surface. Protein adsorption is often driven by processes leading to an increase in entropy. An important process is the dehydration of a hydrophobic protein and/or sorbent surface. In addition, perturbations in the protein structure, leading to more rotational mobility around the polypeptide backbone, can provide a dominant entropic contribution (Haynes and Norde, 1995; Norde, 1996). In general, a decrease in the amount of $\alpha$-helix and $\beta$-sheet is found upon interaction of proteins with hydrophobic surfaces (Wu et al., 1993; Kondo et al., 1992). The increase in $\alpha$-helical content upon adsorption of SC3 is probably an indication of a specific conformational change, which is needed for the adhesive properties of hydrophobins.

After deglycosylation the strong interaction with a hydrophobic solid surface is decreased, as indicated by the removal of the 72% of the adsorbed protein from the Teflon and a correspondingly strong decrease of the ATR-FTIR signal after heating the germanium plate in 2% SDS. The CD spectrum shows that the induction of $\alpha$-helix upon interaction of the deglycosylated protein with the colloidal Teflon is less pronounced than with glycosylated SC3. This suggests that the mannose residues are important for the formation of $\alpha$-helix and the strong interaction with the hydrophobic substrate. Because the mannose residues appear to be situated on the hydrophilic side of the protein film, away from the protein/hydrophobic surface interface, their role in strong protein/hydrophobic surface interactions is probably indirect, via the induction of $\alpha$-helix.

As indicated in Fig. 8, the prediction for $\alpha$-helical structure is low for the entire sequence. The same prediction for COH1 from *Coprinus cinereus*, the hydrophobin most homologous to SC3 (Ásgeirsdóttir et al., 1997), revealed a possible $\alpha$-helical structure for residues 76–86 in COH1. This sequence is predicted to be a $\beta$-sheet structure for SC3, whereas only 2 of the 11 residues are different between the two sequences. Interestingly, this is the only stretch in the entire sequence of SC3 that fits in a helical wheel, with hydrophobic residues spanning about two-thirds of the helix and small or hydrophilic residues at the other side (Fig. 1). These observations make this part of the protein a good candidate for the position of the conformational change into $\alpha$-helix upon assembly on a hydrophobic solid. This helix may form an anchor that binds the protein strongly to hydrophobic surfaces. Studies are in process to address this issue.

## Formation of the rodlet layer

The typical pattern formed after drying down a solution of SC3 consists of a rodlet layer with a thickness of ~10 nm. This is roughly the thickness of a rodlet as detected by electron microscopy. The same layer is found on the outside of aerial hyphae of the fungus (Wösten et al., 1993, 1994b). The rodlets as detected by SFM have a larger diameter, but this could be an effect of tip-broadening during scanning. The normal diameter of a globular protein with the molecular mass of SC3 would be ~3.1 nm, which means that one globular molecule is not enough to span the entire layer. The amphipathic character of the rodlet layer should, therefore, originate from a layer of at least two molecules, or a molecule with an elongated shape, exposing the mannose residues toward the hydrophilic side (Wösten et al., 1994a) and hydrophobic groups (presumably as an $\alpha$-helix) toward the hydrophobic rodlet side.

Hydrophobins can be divided into Class I and Class II hydrophobins, based on the solubility after assembly (Wessels, 1994). The assemblages of Class I hydrophobins are insoluble in hot SDS, whereas the assemblages of Class II hydrophobins can be dissolved in, for example, 60% ethanol. Furthermore, fewer amino acids separate the third and fourth cysteine residue in Class II hydrophobins than in Class I hydrophobins (Fig. 1). Until now the rodlet structures have been found only in Class I hydrophobins (Wessels, 1997). The amphipathic $\alpha$-helix probably induced upon binding of SC3 to a hydrophobic solid is located between the third and fourth cysteine residues. All Class I hydrophobins that form rodlets contain a similar amphipathic $\alpha$-helix at this position in the sequence. When the short stretch between the third and fourth cysteines of Class II hydrophobins is fitted in a helical wheel, an amphipathic helix can also be formed, but this helix is much shorter (up to seven amino acids). This suggests that the $\alpha$-helix is

important for the formation of rodlets. The $\alpha$-helix seems to be typical for Class I hydrophobins and might be involved in the high insolubility of the assemblages and the binding of fungal structures to hydrophobic substrates.

All hydrophobins studied so far have a major effect on the water surface tension. Recently it was shown that fungal structures need the lower surface potential induced by hydrophobins to escape the water phase and grow into the air (Wösten and Wessels, 1997). The induction of $\beta$-sheet at the water-air interface, as shown by ATR-FTIR and CD spectroscopy, seems to be related to this function of hydrophobins. Another conformation, the $\alpha$-helical structure, seems to be involved in the attachment of hyphae to hydrophobic surfaces. In the future we will study the assembly of other hydrophobins to relate the structure in the different states to the function of these hydrophobins.

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