Hydrophobins, the fungal coat unravelled

Han A.B. Wösten [a,*], Marcel L. de Vocht [b,c]

[a] *Groningen Biotechnology and Biomolecular Sciences Institute, Department of Microbiology, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands*
[b] *Groningen Biotechnology and Biomolecular Sciences Institute, Department of Molecular Plant Biology, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands*
[c] *Groningen Biotechnology and Biomolecular Sciences Institute, Department of Biochemistry, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands*

Abstract

Hydrophobins are among the most surface active molecules and self-assemble at any hydrophilic–hydrophobic interface into an amphipathic film. These small secreted proteins of about 100 amino acids can be used to make hydrophilic surfaces hydrophobic and hydrophobic surfaces hydrophilic. Although differences in the biophysical properties of hydrophobins have not yet been related to differences in primary structure it has been established that the N-terminal part, at least partly, determines wettability of the hydrophilic side of the assemblage, while the eight conserved cysteine residues that form four disulphide bridges prevent self-assembly of the hydrophobin in the absence of a hydrophilic–hydrophobic interface. Three conformations of class I hydrophobins have been identified: the monomeric state, which is soluble in water, the α-helical state, which is the result of self-assembly at a hydrophobic solid, and the β-sheet state, which is formed during self-assembly at the water–air interface. Experimental evidence strongly indicates that the α-helical state is an intermediate and that the β-sheet state is the end form of assembly. The latter state has a typical ultrastructure of a mosaic of 10 nm wide rodlets, which have been shown to resemble the amyloid fibrils. © 2000 Elsevier Science B.V. All rights reserved.

1. Introduction

Hydrophobins are small secreted proteins that fulfil a broad spectrum of functions in fungal growth and development. They are involved in formation of hydrophobic aerial structures like aerial hyphae, spores and fruiting bodies (e.g. mushrooms or brackets) [1–3] and mediate attachment of hyphae to hydrophobic surfaces [4] and signalling thereof [5]. The latter is important in initial steps of fungal pathogenesis where the fungus must attach to the hydrophobic surface of the host before penetration and infection can occur [5]. Based on their hydropathy patterns and their solubility characteristics, Wessels [6] discriminated between class I and class II hydrophobins. Despite the fact that the amino acid sequences of hydrophobins within these classes are diverse [1], at least class I hydrophobins seem to be functionally related, i.e. they can (partially) substitute for each other [7,8].

The most characteristic feature of hydrophobins is that they self-assemble at hydrophilic/hydrophobic

* Corresponding author. Fax: +31-50-3632154;
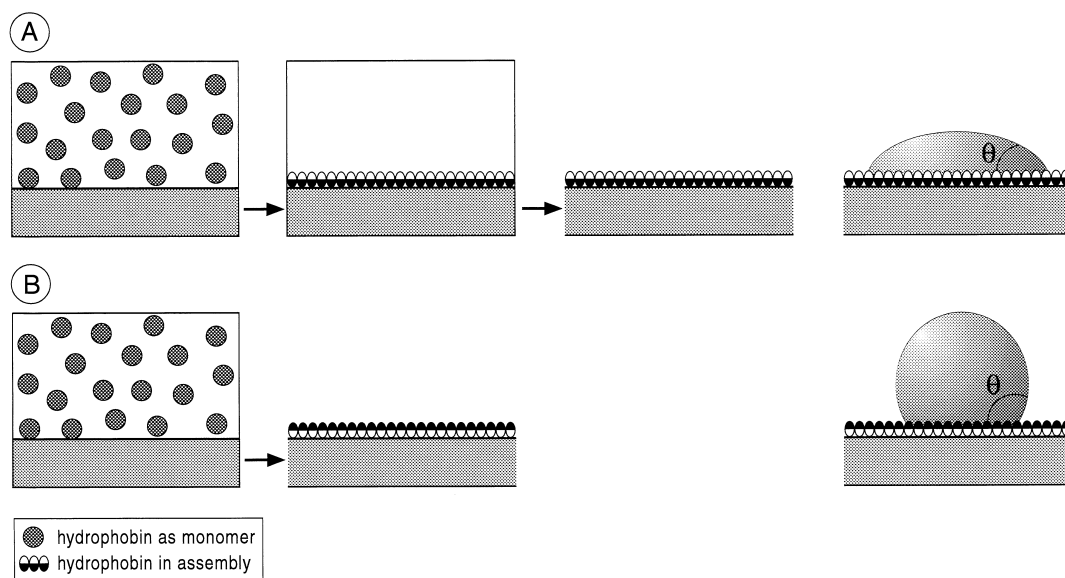E-mail: wostenha@biol.rug.nl

Fig. 1. By self-assembly hydrophobins can change the nature of a surface. (A) A sheet of hydrophobic plastic (e.g. Teflon) immersed in an aqueous solution of a class I hydrophobin becomes coated with a 10 nm thick strongly adhering protein film that makes the surface wettable (water contact angle, θ, ranging between 22–63°). (B) Hydrophobin monomers dried down on a hydrophilic surface (e.g. glass or filter paper) make the surface hydrophobic (water contact angle 110°).

interfaces [4,9,10]. By self-assembly at the interface between the hydrophilic cell wall and a hydrophobic environment (the air or a hydrophobic solid like the surface of a host), emergent structures are covered with an amphipathic membrane [4,11]. The hydrophilic side of this membrane faces the cell wall, while the hydrophobic side is exposed. Aerial hyphae thus become hydrophobic, while hyphae that grow over a hydrophobic substrate can attach themselves to this surface. In this overview we will elaborate on the molecular and biophysical properties of hydrophobins that not only play a crucial role in fungal growth and development but that are also interesting candidates for use in medical and technical applications.

2. Interfacial self-assembly of hydrophobins

Hydrophobins self-assemble at hydrophilic–hydrophobic interfaces (e.g. between water and air, water and oil, or water and a hydrophobic solid like Teflon) into an amphipathic membrane [4,9,10,12–19]. The membranes formed by class I hydrophobins (e.g. SC3 and SC4) are highly insoluble (even resisting 2% SDS at 100°C) and can only be dissociated

with formic acid (FA) or trifluoroacetic acid (TFA) [20–22]. Assemblages formed by class II hydrophobins are less stable. Those of cerato-ulmin (CU) and cryparin (CRP) readily dissociate in 60% ethanol and in 2% SDS [17,19], while assembled CU also dissociates by applying pressure or by cooling [17].

The hydrophilic side of a hydrophobin membrane has a water contact angle ranging between 22 and 63°, while the hydrophobic side exhibits a water contact angle of about 110° (which is similar hydrophobic as Teflon) (Table 1). By self-assembly, hydrophobins can change the nature of a surface. Glass and filter paper turn hydrophobic by drying down a solution of hydrophobin on these surfaces (Fig. 1B) [9,12–14]. Conversely, hydrophobic solids (e.g. Teflon) (Fig. 1A) or oil droplets can be made hydrophilic by submerging or suspending these materials into a solution of hydrophobin [4,10,12–14]. The membrane formed by the class II hydrophobin CRP on a hydrophobic surface is readily removed with SDS or 60% ethanol. In contrast, the interaction of class I hydrophobins with hydrophobic solids is very strong. For instance, the membrane resists washes with water or 2% SDS at 100°C [4,10,13,14]. Class I hydrophobin assembled on filter paper also resists these washes showing that the hydrophilic

Table 1

physiochemical properties of the class I hydrophobins SC3 ([2,4,8,9]; this report), SC4 [8,14], ABH1 and ABH3 [12,13], the class II hydrophobins CRP (this report, [19]) and the tripartite hydrophobin CFTH1 [23]

Hydrophobin	Surface activity (mJ m^{-2})	Hydrophilicity hydrophilic side (θ)	Hydrophobicity hydrophobic side (θ)	Lectin activity	Rodlets
Class I					
SC3	32	36 ± 3	117 ± 8	yes	yes
SC4	35	48 ± 3	115 ± 3	yes	yes
ABH1	ND	63 ± 8	113 ± 4	ND	yes
ABH3	37	59 ± 5	117 ± 3	ND	yes
Class II					
CRP	32	22 ± 2	≥ 90[a]	yes	no
CFTH1	33	60 ± 5	105 ± 2	ND	no

Hydrophilicity and hydrophobicity of both sides of the hydrophobin membrane are expressed as the water contact angle (θ). ND, not determined.
[a] It was not possible to obtain a homogenous hydrophobic coating.

side can also strongly interact with a solid, possibly due to a lectin-like activity (see Table 1).

Hydrophobins belong to the most surface active molecules. With a maximal lowering of the water surface tension from 72 mJ m^{-2} to 24 mJ m^{-2} at 50 µg ml^{-1}, SC3 is the most surface active protein known [3]. Other hydrophobins are also highly surface active ([13,14,23], Table 1). Their activity is at least similar to that of traditional biosurfactants encompassing glycolipids, lipopeptides/lipoproteins, phospholipids, neutral lipids, substituted fatty acids, and lipopolysaccharides (for reference see [24]). However, in contrast to these surfactants, surface activity of hydrophobins is not dependent on a lipid molecule but is solely caused by the amino acid sequence (see below). Interestingly, SC3 hydrophobin monomers seem not to be highly surface active. Surface activity is attained by a conformational change that occurs when SC3 monomers self-assemble at the water–air interface [25,26].
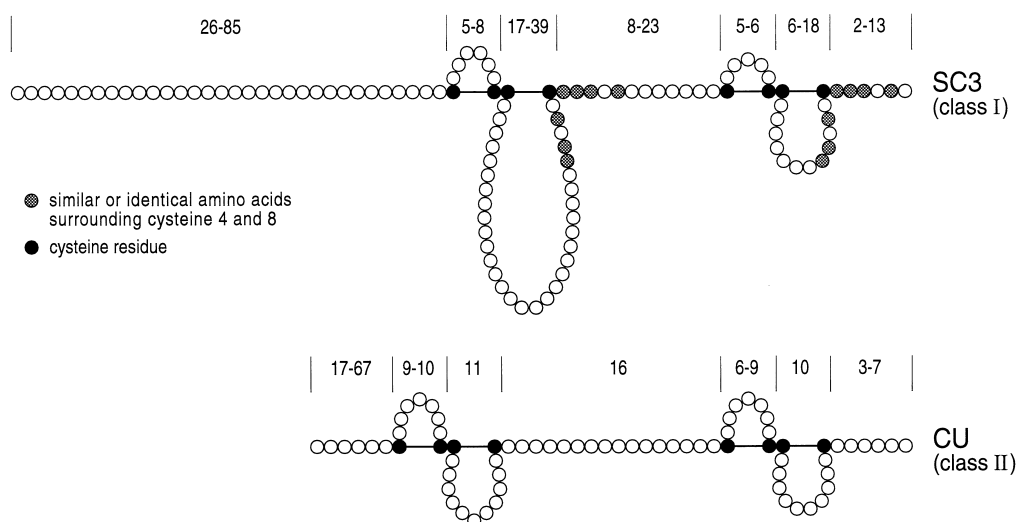

Fig. 2. Cysteine linkages of CU [27] and the length of the sequences spacing the cysteine residues in class I and class II hydrophobins. Cys1 of CU interacts with Cys2 or 3, and Cys2 or 3 is linked to Cys4. Similarly, Cys5 interacts with Cys6 or 7, while Cys6 or 7 is linked to Cys8. The spacing of the cysteine residues, the hydropathy patterns, and the similarity of the most prevalent amino acids surrounding the fourth and eighth cysteine residue of most class I hydrophobins suggest that hydrophobins are two-domain proteins. Note that the indicated length of the N-terminal part of the class I and class II hydrophobins preceding the first cysteine residue includes the signal sequence (± 20 aa) that is absent in the mature proteins.

The properties of hydrophobins make them interesting candidates for use in medical and technical applications [1]. For instance, they could be used in tissue engineering to increase biocompatibility of hydrophobic surfaces or to prevent bacterial adhesion to catheter surfaces. Hydrophobins may also be used as an intermediate to attach cells or molecules to hydrophobic surfaces as in biosensors. Of course, a hydrophobin should meet certain requirements to make them optimally suited for a specific application. Nature produces a variety of hydrophobins, each with slightly different properties. Alternatively, hydrophobins could be modified by chemical cross-linking or genetic engineering.

3. Primary structure of hydrophobins

Hydrophobins are small (±100 aa) secreted proteins characterised by eight conserved cysteine residues and a conserved spacing of hydrophilic and hydrophobic regions [1]. However, their amino acid sequences are diverse [1,6].

The cysteine residues of the class I hydrophobin SC3 [22] and the class II hydrophobin cerato-ulmin (CU) [27] form intramolecular disulphide bridges. The interacting cysteine residues in CU were determined [27] (Fig. 2). Assuming that disulphide bridges in class I hydrophobins are identical to those of CU, both hydrophobin classes seem to contain two domains. Cysteine 1–4 is contained in the first domain, while the second domain encompasses cysteine 5–8 (Fig. 2). The presence of two domains is also indicated by the hydropathy patterns [28] and by the similarity of the most prevalent amino acids surrounding the fourth and eighth cysteine residue of most class I hydrophobins [2]. Differences in the biophysical properties between class I and class II hydrophobins have not yet been related to their primary structure. The most obvious difference is that class II hydrophobins contain more charged amino acids [2].

Mass spectra of the class I hydrophobins SC4 and ABH3 [13,14] and the class II hydrophobin CRP are in good agreement with the masses predicted from their primary amino acid sequence, showing that the remarkable properties of hydrophobins can be solely determined by amino acid residues. Yet, hydropho-
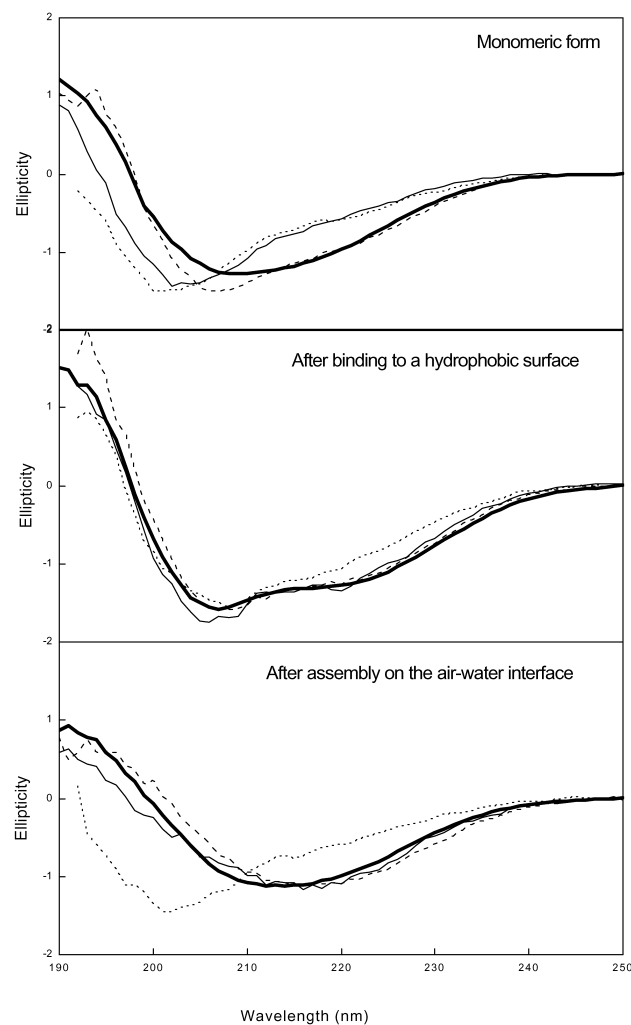

Fig. 3. The secondary structure changes of hydrophobins can be followed by circular dichroism. This figure shows the far-UV region of the circular dichroism spectra of the class I hydrophobins SC3 (thick solid line), SC4 (thin line) and ABH3 (dashed line) and the class II hydrophobin CRP (dotted line) in the monomeric form (upper panel), after addition of colloidal Teflon (middle panel) and after vortexing (lower panel).

bins may be post-translationally modified [26,29]. For instance, the class I hydrophobin SC3 is a glycoprotein containing 16–22 mannose residues that are linked to the N-terminal part of the mature protein ([26]; K. Scholtmeijer and H.A.B. Wösten, unpublished). These mannose residues are exposed at the hydrophilic side of assembled SC3 [30] and were therefore expected to determine surface properties of this side. Indeed, when 26 out of 31 amino acids of the N-terminal part of mature SC3 were
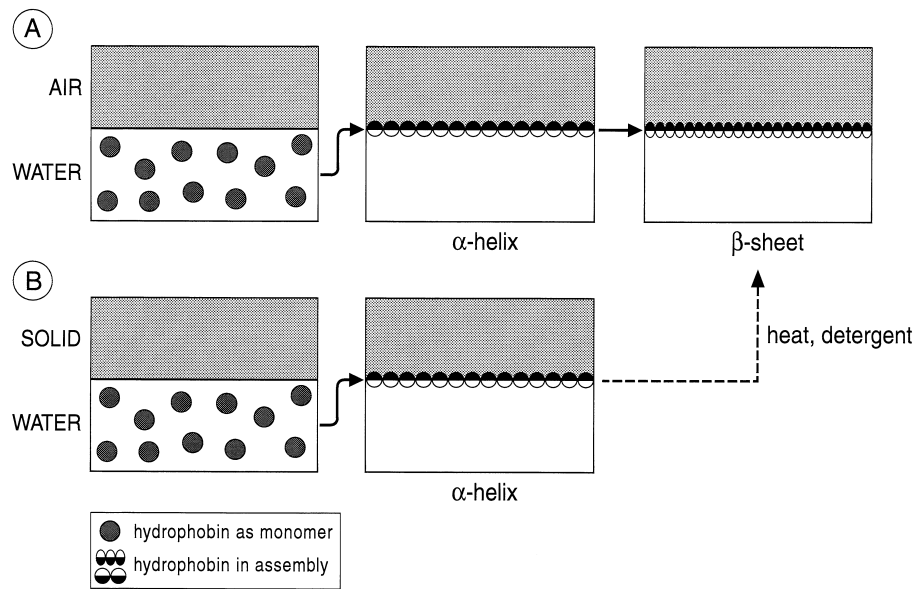
Fig. 4. Model for assembly of the class I hydrophobin SC3. Monomers diffuse to the hydrophilic–hydrophobic interface, where they initially attain the α-helix structure. At the water–air interface the α-helix structure changes to the β-sheet state. At a hydrophobic solid the hydrophobin is arrested in the α-helix structure, unless the coated surface is heated at 100°C in 2% SDS (dashed line). It is not yet known whether self-assembly of class II hydrophobins is accompanied by similar conformational changes.

removed by genetic engineering the protein still assembled but was no longer glycosylated and wettability of the hydrophilic side of the assembled form decreased from 40 to 70° (K. Scholtmeijer and H.A.B. Wösten, unpublished).

Recent findings indicate that hydrophobins have diverged considerably without affecting gross properties of the protein. A remarkable hydrophobin, called CFTH1, was identified that contains three class II hydrophobin-like domains each preceded by a Gly–Asn rich repeat [23]. Interestingly, this large protein behaves like a class II hydrophobin (Table 1). In addition, a gene encoding a class I hydrophobin was identified that contains a leucine zipper domain, which was suggested to act in dimerisation [31]. However, as described above all hydrophobins contain the eight conserved cysteine residues that form four disulphide bridges. When the disulphide bridges of the class I hydrophobin SC3 were reduced and the sulfhydryl groups blocked with iodoacetamide the protein assembled in water in the absence of a hydrophilic–hydrophobic interface [32]. The structure was indistinguisable from that of native SC3 assembled at the water–air interface. Apparently, the disulphide bridges of hydrophobins keep monomers soluble in water (e.g. within the cell or in the medium), allow-

ing self-assembly at a hydrophilic–hydrophobic interface only.

4. Changes in secondary structure upon self-assembly of hydrophobins

The secondary structure of monomeric and assembled hydrophobins was determined with infrared spectroscopy and circular dichroism spectroscopy ([26,27]; Fig. 3). Monomers of the class I hydrophobins SC3, SC4 and ABH3 and the class II hydrophobin CU are rich in β-sheet structure ([23,26]; Fig. 3). Upon self-assembly, hydrophobins change their structure. At least for class I hydrophobins, this change depends on the nature of the hydrophilic–hydrophobic interface. After assembly at the water–air interface (i.e. by vortexing the aqueous hydrophobin solution) all class I hydrophobins studied attain more β-sheet structure indicated by the minima at 215–217 nm. However, at the interface between water and a hydrophobic solid such as Teflon, α-helical structure is induced as can be concluded from the characteristic increase in intensity around 192, 205 and 220 nm ([26]; Fig. 3). Both assembled forms of class I hydrophobins have an amphipathic

nature and can be dissociated with TFA, which unfolds the protein completely as was shown by one-dimensional (1D) NMR. After removing the solvent and adding water, the class I hydrophobins refold to the same monomeric structure that was observed before purification or TFA treatment [26] and the process of self-assembly can be repeated [9,12]. Similar to class I hydrophobins, self-assembly and disassembly of class II hydrophobins can be cycled repeatedly even after dissociation of the membrane by TFA.

Self-assembly of the class I hydrophobin SC3 appears to be a multi-stage process (M.L. de Vocht, unpublished; Fig. 4). At the water–air interface monomers initially attain an intermediate form, which we think is similar to the α-helical form observed at the interface between water and a hydrophobic solid. At 5 μg ml^{-1}, this form of SC3 is observed within a few minutes and during approximately 2 h the conformation changes to the β-sheet structure. SC3 organised into the characteristic rodlet structure (see below) is in this β-sheet structure. The α-helical form of SC3 as observed at the interface between water and a hydrophobic solid can also be converted to the β-sheet form. However, this conformational change only occurs at 100°C in the presence of 2% SDS (M.L. de Vocht, unpublished). These data strongly indicate that the β-sheet structure of SC3 is the stable end form and that upon assembly at the water–Teflon interface SC3 is arrested in the intermediate α-helix structure. This arrest would be released by the combination of the detergent and heat. It is not yet shown which structure SC3 attains at the interface between water and an apolar liquid. Rodlets were observed at the water–oil interface [4], suggesting that SC3 can attain its β-sheet structure at this interface. It was proposed that residues 76–86 of SC3 could form an amphipathic α-helix [26]. This part of the sequence could act as a sensor that triggers subsequent polymerisation events after interaction with a hydrophilic–hydrophobic interface.

The class II hydrophobin CRP also attained more α-helix structure at the interface between a hydrophobic solid and water (Fig. 3), although this change was less pronounced. A structural change at the water–air interface could not be observed because of the instability of the assemblages. Therefore it is not yet known whether the structural changes ob-
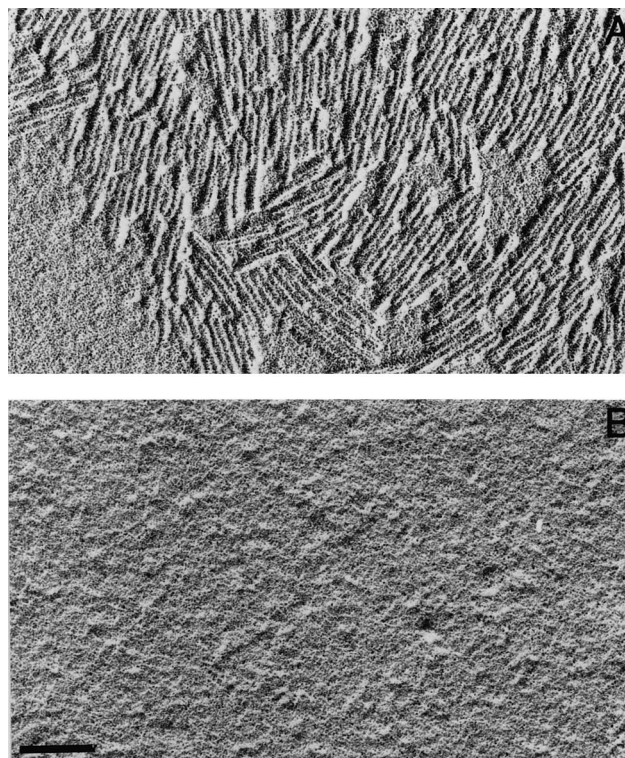

Fig. 5. The hydrophobic side of class I hydrophobins is characterised by a mosaic of rodlets as shown by surface shadowing. In contrast, no rodlets are observed in the case of the class II hydrophobin CRP.

served during self-assembly of class I hydrophobins also apply for class II.

The above studies were all performed at a static hydrophilic–hydrophobic interface. Self-assembly was observed at concentrations lower than 1 μg/ml. By rotating an aqueous solution a dynamic water–air interface is created. Under this condition SC3 only assembled above a concentration of 3.4 μg ml^{-1} (at 26°C) (M.L. de Vocht, unpublished). This critical concentration indicates that self-assembly of hydrophobins follows a nucleation dependent polymerisation mechanism as was shown for amyloids [33] and other polymerising proteins like actin [34] and tubulin [35]. The critical concentration above which SC3 self-assembles decreases with increasing temperature, indicating that self-assembly is an endothermic process. Therefore, self-assembly must be driven by an increase in entropy, probably by hydrophobic interactions since changes in the pH or the presence of salt hardly affected self-assembly.

5. Ultrastructure of assembled hydrophobins

The hydrophobic side of SC3 and other class I hydrophobins is characterised by a mosaic of 5–12 nm wide parallel rodlets (Fig. 5) [4,5,7,9,12–14,36–38]. Atomic force microscopy showed that the rodlets of SC3 consist of two tracks of 2–3 protofilaments with a diameter of about 2.5 nm each (M.L. de Vocht and I. Reviakine, unpublished). No rodlet pattern was seen at the hydrophobic side of the class II hydrophobin CRP (Fig. 5) and the tripartite hydrophobin CFTH1 [23]. Whether the absence of rodlets or the differences in rodlet diameter has any functional consequences is not yet known.

The rodlets of class I hydrophobins are very similar to the fibrils formed by amyloid proteins. They are composed of 4–6 protofilaments with similar diameters, have a high amount of β-sheet structure, are protease resistant, self-assemble via intermediates, and assemble only above a critical concentration ([26,39–41]; M.L. de Vocht unpublished). In addition, assembled SC3 and SC4 increase thioflavine T (ThT) fluorescence and change the absorption spectrum of Congo red (M.L. de Vocht, unpublished). These criteria are generally used to monitor amyloid formation [42,43] and therefore strongly indicate that hydrophobin rodlets are amyloid fibrils. Interestingly, other amyloid proteins can also self-assemble on a hydrophobic surface [44] or the air–water interface [45]. The polymerisation in response to interfaces may be a general property of amyloid proteins and might be important for self-assembly in vivo. In the cell the self-assembly could occur on biological surfaces such as membrane interfaces or protein colloids.

It was suggested that amyloid fibril formation is common to many, if not all, polypeptide chains [46–48]. There are many diseases related to amyloid formation and in these cases the fibril formation is malignant for the organism [39]. In contrast, hydrophobins seem to be evolved to form amyloid-like structures at hydrophilic–hydrophobic interfaces in a way that is beneficial to the fungus. To our knowledge hydrophobins are the first example of a functional amyloid protein. Similar to other amyloid forming proteins (see [49]), hydrophobins could have attained an increased propensity to form fibril-

lar structures by one or two mutation(s) in a protein that served a different function.

Unfortunately, until now no tertiary structure of hydrophobins is available. We have tried to use NMR and 3D reconstructions by Fourier transform analysis to resolve the structure of monomeric and assembled SC3, respectively. However, NMR spectra indicated that monomeric SC3, either or not stabilised with SDS, ethanol or DMSO is not highly structured. This may be confirmed by hydrogen/deuterium exchange experiments. Possibly, hydrophobins become more structured after self-assembly. Yet, 3D reconstruction using negatively stained hydrophobin and Fourier transform analysis indicated that the assemblages are not highly ordered. More information on the structure of the rodlet might be obtained with solid state NMR.

6. Conclusions

Hydrophobins are remarkable proteins that self-assemble at hydrophobic–hydrophilic interfaces. Studying their biophysical properties helped us to understand their role in fungal growth and development [1–3,28] and indicated their potential use in medical and technical applications [1]. The 3D structure of both monomeric and assembled hydrophobin is not known yet but the assemblages of at least the SC3 and SC4 hydrophobins resemble those of amyloid proteins [41].

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