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Atomic Composition of the Hydrophobic and Hydrophilic Membrane Sides of Self-Assembled SC3p Hydrophobin

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The hydrophobin SC3p of *Schizophyllum commune* self-assembles into a 10-nm-thick amphipathic membrane at hydrophilic-hydrophobic interfaces. X-ray photoelectron spectroscopy of the hydrophobic membrane side of SC3p, assembled in vitro, showed an atomic composition similar to the calculated composition of SC3p when glycosylation was taken into account. The atomic composition measured at the hydrophilic membrane side deviated from that at the hydrophobic side and indicated the presence of a lower number of peptide bonds. High levels of S and N were detected only on mycelia carrying hydrophobic aerial hyphae, as expected with assembled SC3p present at the surface of these hyphae.

Hydrophobins, involved in fungal development, are small excreted proteins rich in hydrophobic amino acids and sharing similar hydropathy plots when the eight conserved cysteine residues are aligned (4). They were discovered as the products of a gene family highly expressed during development of aerial hyphae and fruit bodies in the homobasidiomycete *Schizophyllum commune* (2, 5). The hydrophobin encoded by *SC3*, SC3p, self-assembles at hydrophilic-hydrophobic interfaces into an amphipathic protein membrane that is typified at its hydrophobic side by a mosaic of 10-nm-wide rodlets (7–10). Monomeric SC3p is excreted into the medium by submerged hyphae, but the protein self-assembles at the wall-air interface of aerial hyphae and is responsible for the high hydrophobicity of the surface of these hyphae (7, 8). A mutant carrying the allele *thn* forms few aerial hyphae and does not produce hydrophobins (6).

In the present study, we used X-ray photoelectron spectroscopy (XPS) to initiate studies of the molecular basis of the amphipathic character of the SC3p membrane by examining surfaces on which SC3p assembled. XPS is based on irradiating a surface with X-rays and analyzing the kinetic energy of the photoejected electrons. This provides an elemental surface analysis with an analyzed depth in the nanometer range. Since the photoelectron kinetic energy is dependent on the chemical state of the element, different chemical functional groups present at the sample surface can be discriminated. XPS is a familiar technique in the field of materials science but is nowadays also applied to microorganisms (1).

A monokaryotic wild-type strain of *S. commune* and a derived *thn* mutant (6) were grown from mycelial homogenates spread on top of a polycarbonate membrane positioned on solid medium (7). At various times, mycelial mats were harvested and washed with water, and portions were dried on gold-coated coverslips with the upper surface facing the air. This surface was used for water contact angle measurements, as a measure of hydrophobicity (3), and for XPS analysis.

After 3 days of culturing, the wild-type strain developed aerial hyphae known to contain assembled SC3p at their surfaces (7). At that time, the moderately hydrophilic surface of the mycelium (water contact angles of about 40°) developed hydrophobicity, which continued to increase up to water contact angles of 125° on day 6. During the formation of aerial hyphae, XPS of the mycelial surface showed significant increases of the N/C ratio ($P < 0.005$) from 0.08 to 0.15 and the S/C ratio ($P < 0.05$) from 0.002 to 0.007, indicating the appearance of proteins rich in S, such as SC3p. However, these values remain lower than those measured at the hydrophobic side of in vitro-assembled SC3p (Table 1), probably because of the presence of nonproteinaceous material covering the rodlet layer of aerial hyphae (8). XPS of the mycelial mats of the *thn* mutant, which did not develop aerial hyphae or increase in hydrophobicity, showed low N/C ratios (0.04 to 0.05), while sulfur was not detected. This indicates a small amount of protein at the surfaces of these hyphae, reflecting the absence of SC3p in these mycelia (6).

By evaporation of an aqueous solution of SC3p, the hydrophobin assembles at the water-air interface, exposing its hydrophobic membrane side (7, 8). On the other hand, by immersing a hydrophobic polymer in an aqueous solution of SC3p, the hydrophilic side of the SC3p membrane is exposed as a result of assembly at the (hydrophilic) water-(hydrophobic) polymer interface (9, 10).

XPS showed that after drying down of 100 μl of an aqueous solution of SC3p (50 μg ml^{-1}) on polytetrafluoroethylene (PTFE-Teflon) the measured F/C ratio (theoretically 2 for PTFE-Teflon) was reduced from 2.3 to 0.03, while after drying down on gold-coated glass no gold-specific electrons could be detected, indicating complete or nearly complete coverage of the surfaces with SC3p. Irrespective of whether the source of SC3p was medium or cell walls, XPS showed an atomic composition of the exposed hydrophobic membrane side (water contact angles of 95°) similar to that predicted from the known amino acid composition of SC3p (Table 1). Only the O/C ratios were higher than the value calculated for the polypeptide chain. This is probably due to the presence of O-linked mannose residues in this glycoprotein (data not shown). By assuming attachment of 11 anhydrohexose residues ($C_{66}H_{110}O_{55}$) per polypeptide chain, calculated and experimental O/C and also N/C and S/C ratios matched closely (Table 1). The distribution of carbon

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TABLE 1. Experimental and calculated atomic compositions of SC3p assembled in vitro

Surface	Ratio ± SD[a]						
	O/C	N/C	S/C	N/S	C—(C,H)/C	C—(O,N)/C	C═O/C
Hydrophobic side (SC3p from cell wall)	0.41 ± 0.01	0.22 ± 0.01	0.015 ± 0.001	14 ± 1	0.38 ± 0.05	0.39 ± 0.01	0.23 ± 0.01
Hydrophobic side (SC3p from medium)	0.41 ± 0.01	0.23 ± 0.01	0.015 ± 0.003	15 ± 3	0.37 ± 0.04	0.35 ± 0.02	0.28 ± 0.05
Hydrophilic side (SC3p from medium)	0.42 ± 0.04	0.16 ± 0.02	0.011 ± 0.001	15 ± 3	0.47 ± 0.04	0.34 ± 0.02	0.19 ± 0.02
Mature polypeptide[b]	0.34	0.27	0.019	14	0.39	0.34	0.27
Mature polypeptide + carbohydrate $(C_6H_{10}O_5)_{11}$	0.41	0.23	0.016	14	0.33	0.43	0.23

[a] The upper three rows indicate experimental values, and the lower two rows indicate the calculated compositions of nonmodified and modified proteins. Standard deviations are based on at least six measurements involving two independently isolated preparations.
[b] Calculated from data of Schuren and Wessels (2) and Wessels et al. (5).

bound to carbon or hydrogen only [C—(C,H)], carbon singly bound to oxygen or nitrogen [C—(O,N)], and carbon doubly bound to oxygen (C═O) was also in close agreement with the expected values (Table 1).

The hydrophilic side of the SC3p membrane was exposed by immersing PTFE-Teflon sheets overnight in a glass cuvette containing an aqueous solution of 50 μg of SC3p ml^{-1}, decreasing water contact angles at the surface from 108 to 48° (10). XPS showed that coating with SC3p caused a drop in F/C ratios from 2.3 to 0.1, indicating nearly complete coverage of the Teflon with assembled SC3p. At the hydrophilic side of assembled SC3p, the N/C ratio ($P < 0.005$) and the S/C ratio ($P < 0.05$) were significantly lower than at the hydrophobic membrane side, while the N/S ratio was similar (Table 1). Also, the C═O/C ratio was lower ($P < 0.005$) and the C—(C,H)/C ratio was higher ($P < 0.005$). At both sides of the membrane, the ratios of C═O/C correspond to that of N/C, which is expected because of a prevalence of nitrogen in peptide bonds.

Since experimental XPS values obtained at the hydrophobic side of assembled SC3p were similar to those expected for the whole protein (2, 5), emitted photoelectrons must have originated from all parts of the 10-nm-thick membrane. However, only few electrons are expected to be emitted from a depth exceeding 5 nm after excitation by X rays (1). Possibly, the serrated structure of the hydrophobic membrane side caused by the presence of rodlets in a random orientation allows for the emission of photoelectrons from all parts of the SC3p membrane. In contrast, XPS at the smooth hydrophilic membrane side of assembled SC3p (10) would detect photoelectrons emitted from only part of the membrane. The low N/C, S/C and C═O/C ratios at this side indicate an orientation of peptide bonds and amino acid chains towards the hydrophobic side of the membrane and possibly sugar residues oriented towards the hydrophilic side.

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