A Structural and Biochemical Model of Processive Chitin Synthesis*

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Background: Chitin synthesis is an attractive drug target in a range of organisms but is not understood at the molecular level.

Results: The chitooligosaccharide synthase NodC can be assayed with a novel HTS assay, and the mechanism/fold can be probed by site-directed mutagenesis and topology mapping.

Conclusion: NodC is a model system to probe chitin synthesis.

Significance: This work enables the exploitation of chitin synthesis as a drug target.

Chitin synthases (CHS) produce chitin, an essential component of the fungal cell wall. The molecular mechanism of processive chitin synthesis is not understood, limiting the discovery of new inhibitors of this enzyme class. We identified the bacterial glycosyltransferase NodC as an appropriate model system to study the general structure and reaction mechanism of CHS. A high throughput screening–compatible novel assay demonstrates that a known inhibitor of fungal CHS also inhibits NodC. A structural model of NodC, on the basis of the recently published BcsA cellulose synthase structure, enabled probing of the catalytic mechanism by mutagenesis, demonstrating the essential roles of the DD and QXXRW catalytic motifs. The NodC membrane topology was mapped, validating the structural model. Together, these approaches give insight into the CHS structure and mechanism and provide a platform for the discovery of inhibitors for this antifungal target.

Fungal infections are a threat to human health worldwide. Fungal cells are protected by a unique cell wall, which is a dynamic structure consisting of proteins and polysaccharides. One essential component is chitin, a β-1,4-linked polymer of GlcNAc. Because chitin is absent in vertebrates, its synthesis is a promising target for developing specific drugs against fungal infections. Chitin synthases (CHS)3 utilize the nucleotide sugar donor UDP-GlcNAc and transfer the α-linked GlcNAc sugar in an inverting mechanism onto the non-reducing end of the growing acceptor oligosaccharide (1). CHS enzymes are classified in the CAZy database as belonging to the GT-2 family (2). This family contains inverting GTs such as CHS, cellulose syntheses, and hyaluronan synthases. Furthermore, CHS have been categorized into classes I, II, and III according to sequence conservation (3). In Saccharomyces cerevisiae, three chitin synthases have been identified and characterized functionally by means of gene disruption. These studies revealed that although single disruption of any of the three S. cerevisiae chs genes does not affect viability, the combined deletion of chs2 and chs3 is lethal (4).

CHS are large, membrane integrated enzymes with multiple domains important for subcellular localization and activation. CHS contain multiple transmembrane (TM) domains that are thought to form a transport channel for the deposition of chitin on the outer membrane, similar to cellulose synthases (5). In yeast, the best characterized CHS enzyme is chitin synthase 2 (ScCHS2). This enzyme consists of three domains: an N-terminal domain, a catalytic domain of the GT-2 family containing a GT-A fold, and a C-terminal transmembrane domain. ScCHS2 activity appears to be regulated by proteases or posttranslational modifications (6, 7). The N-terminal domain has been shown to be highly phosphorylated in vivo (7). Partial proteolysis with trypsin activates CHS in vitro, releasing shorter CHS fragments lacking the N-terminal domain. Truncation of this domain (d193–ScCHS2) does not affect enzymatic activity (6).

ScCHS2 possesses several conserved sequence motifs that are essential for chitooligosaccharide synthesis. Nagahashi et al. (8) have identified a conserved region upstream of the first predicted TM domain. CON1 (ScCHS2 residue range 490–607) contains the sequence motifs D, (E/D)DX, and Q(R/Q)XRW, which are essential for catalytic activity. Interestingly, CON1 is conserved not only among class II CHS but also found in chitooligosaccharide synthases such as the bacterial NodC proteins found in Rhizobium sp. and the DG42 protein from Xenopus (9, 10). A second conserved CHS class II region (CON2, ScCHS2 residue range 748–815) is indispensable for the synthesis of long chitooligosaccharides because mutations of single residues in this region affect the ability of ScCHS2 to...
synthesize GlcNAc oligomers longer than chitobiose (10). CON2 is predicted to be in the cytosol after the first two TM domains (10).

Several recent reviews have covered advances in the targeting of CHS for antifungal drug development (11, 12). In 1991, Cabib (13) tested the natural product inhibitors Polyoxin D, Nikkomycin Z, and Nikkomycin X and showed these to be competitive inhibitors of CHS2. These compounds possess a chemical scaffold similar to the substrate UDP-GlcNAc and are, therefore, believed to compete for the UDP-GlcNAc binding site in the active site of CHS (12). In 2000, a series of new CHS1 inhibitors were identified by high throughput screening and optimized by systematic chemical modifications (14). This strategy resulted in the most potent non-competitive chitin synthase inhibitor known to date, RO-09-3024, showing an IC50 of 0.14 nM in vitro and an EC50 of 0.07 mg/ml versus the human pathogen Candida albicans (CY1002) (15). Since then, in essence, the pursuit of CHS inhibitors has proceeded only by exploring existing chemical space because the structures of the binding modes of existing compounds remain unknown. Structural insights into the CHS active site, combined with inhibitor screening, would give rise to new opportunities to advance these existing scaffolds in antifungal drug development.

CHS are multitransmembrane proteins that, to date, have resisted protein expression, solubilization, and crystallization for structural studies or high throughput ligand screening. A possible solution to this is to identify bacterial homologues of CHS that contain fewer transmembrane domains, are smaller, do not require eukaryotic posttranslational modifications, and can, therefore, be expressed in bacterial systems. One such apparent orthologue is the rhizobial enzyme NodC, a processive glycosyltransferase that synthesizes the chitooligosaccharide backbone of the rhizobial nodulation factor (Nod factor) essential for root nodulation of legumes (16). NodC is a \(1,4-N\)-acetylglucosamine transferase that utilizes UDP-GlcNAc as a nucleotide sugar donor and GlcNAc as the acceptor sugar to processively synthesize the Nod factor backbone, a chitooligosaccharide. NodC enzymes from different rhizobial species synthesize chitooligosaccharide backbones of specific lengths, varying from tri- to pentasaccharides (17 and reviewed in Refs. 18, 19). NodC enzymes possess striking sequence conservation with the catalytic core of CHS enzymes (20, 21). NodC proteins are smaller than CHS enzymes (420 amino acids in length compared with 900 to several thousand amino acids in length) because NodC enzymes lack two domains observed in chitin synthases: the N-terminal domain and the...
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C-terminal transmembrane domain that is predicted to form a chitin transport channel across the membrane. However, the catalytic core is conserved (CON1), which contains the conserved CHS motifs (D, (E/D)DX, and Q(Q/R)XRW; Fig. 1). Topology predictions have suggested that NodC enzymes probably contain four transmembrane domains in a structural arrangement similar to that predicted for chitin synthases (22).

Here we show that bacterial NodC is a suitable model to study chitin synthases on a structural and mechanistic level. We demonstrate that NodC is inhibited by a chitin synthase inhibitor. Aided by a structural model exploiting the recently published BcsA structure, we mapped the membrane topology of SmNodC and identified conserved catalytic residues for chito-oligosaccharide synthesis in SmNodC. The structural model provides insights into the molecular mechanism of chitin synthesis.

EXPERIMENTAL PROCEDURES

Cloning and Expression of SmNodC and Mutants—Sinorhizobium melloti NodC (SmNodC) was PCR-amplified and cloned into the pExmCherry plasmid using the BamHI and XhoI sites. Site-directed mutagenesis was performed using the QuikChange method (Stratagene) using standard protocols. All DNA constructs were verified by DNA sequencing (The Sequencing Service, College of Life Sciences, University of Dundee, Scotland, UK). SmNodC-pExmCherry constructs were transformed into Escherichia coli BL21 (DE3) C43 cells. Cells were grown overnight at 37 °C in Luria-Bertani medium containing 50 μg/ml ampicillin. 10 ml of the overnight culture was used for inoculation of 1 liter of Luria-Bertani medium plus ampicillin. The bacteria were grown to an A600 of 0.6, induced by addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside, and cultured for 2 h at 28 °C. Cells were harvested by centrifugation for 25 min at 3300 × g (4 °C). The pellet from 1 liter of culture was washed with 25 ml of chilled H2O and centrifuged for 25 min at 3300 × g (4 °C). The cells were flash-frozen in liquid nitrogen, thawed at room temperature, and resuspended in 25 ml of ice-cold buffer A (25 mM Tris-HCl (pH 7.5), 250 mM NaCl, and 2.5 mM EDTA) supplemented with 1 mg/ml of DNase and half a protease inhibitor tablet (Roche), including 2 mM DTT. The cell pellets were sonicated on ice six times for 30 s each time, with a 1-cm diameter sonicator probe (Sonya Soniprep 150). The fractions were centrifuged twice for 10 min at 12,000 × g, followed by a 60-min spin at 100,000 × g. The membrane fraction was homogenized with a Dounce homogenizer to a concentration of 25 mg/ml in 25 mM Tris-HCl (pH 7.5), 250 mM NaCl, and snap-frozen in liquid nitrogen.

Cloning, Expression, and Investigation of SmNodC-GFP and PhoA Fusion Constructs—Full-length NodC and 11 C-terminal truncation constructs were cloned into GFP (pWaldo-d) and alkaline phosphatase (PhoA) expression vectors. SmNodC-PhoA fusion proteins were expressed in E. coli CC118 cells. For the PhoA activity assay, 5 ml of Luria-Bertani medium + ampicillin was inoculated with 100 μl overnight culture. The cells were grown to an A600 of 0.13–0.16 at 37 °C. 1 ml of this culture was induced with 8 μl of 20% arabinose and grown to an A600 of 0.3–0.5. The cultures were treated with 4 μl of 200 mM iodoacetamide (in 10 mM Tris-HCl (pH 8.0)), incubated for 5 min at room temperature, and spun down for 20 min at 1700 g (4 °C). The cell pellet was washed with 1 ml of buffer (10 mM Tris-HCl (pH 8.0), 10 mM MgSO4, and 1 mM fresh iodoacetamide) and again centrifuged as described previously. The cell pellet was resuspended in 800 μl of buffer (1 mM Tris-HCl (pH 8.0) and 1 mM fresh iodoacetamide). From this, 100 μl were mixed with 900 μl of activity buffer (1 mM Tris-HCl (pH 8.0), 0.1 mM ZnCl2, and 1 mM fresh iodoacetamide). 4 μl of 0.1% SDS and 4 μl of chloroform were added and incubated for 5 min at 37 °C on a shaker (120 rpm). The samples were kept on ice, and 100 μl of 0.4% p-nitrophenyl phosphate were added to each reaction. This reaction was incubated for 90 min at 37 °C, and 100 μl of this solution was pipetted into a clear 96-well plate. Fluorescence was measured at excitation and emission wavelengths of 405 and 550 nm, respectively. All measurements were performed in triplicate.

The SmNodC-GFP-fusion constructs were expressed in E. coli BL21 (DE3) C43 cells. Expression was induced at an A600 of 0.6 at 37 °C, and cells were grown for another 4 h at room temperature. A 1-ml sample of these cultures was centrifuged, and the cell pellet was resuspended in PBS. GFP fluorescence was investigated by in-gel fluorescence (23). All measurements were performed in duplicate.

Enzymology—The steady-state kinetics of WT SmNodC-mCherry fusion were determined using a coupled enzyme assay. UDP-GlcNAc and the fluorogenic substrate 4-methylumbelliferyl-GlcNAc (4MU-GlcNAc) were obtained from Sigma. All measurements were performed in triplicate. Standard reaction mixtures consisted of 25 μM of mixed membrane fractions, 25 mM Tris-HCl (pH 7.5), 250 mM NaCl, and 5% (v/v) glycerol in a total volume of 50 μl, incubated at room temperature (20 °C). The assays were initiated by adding the mixed membrane fractions and stopped after 60 min with 50 μl of a solution containing 25 mM Tris-HCl (pH 7.5), 250 mM NaCl, and 20 mM EDTA. 0.1 μM of Aspergillus fumigatus chitinase B (33) was added to the reaction mixture and incubated for 60 min. The fluorescence of the released 4MU was quantified using an FLX 800 microplate fluorescence reader (Bio-Tek), with excitation and emission wavelengths of 360 and 460 nm, respectively. Metal dependence assays of WT SmNodC were performed as above, with addition of 1 or 10 mM of MgCl2, ZnCl2, CoCl2, NiCl2, or MnCl2. WT activity was standardized to 100%, and background signals were subtracted from WT reactions without UDP-GlcNAc. Reactions were performed in triplicate. The apparent Km and kcat for UDP-GlcNAc and 4MU-GlcNAc were determined by Michaelis-Menten kinetics using varying concentrations of one substrate in the presence of an excess of the other substrate. Data were analyzed in the GraphPad Prism program. SmNodC point mutants were assayed using the protocol developed for WT SmNodC in the presence of 10 mM MgCl2. Nikkomycin Z was purchased from Sigma.

RESULTS AND DISCUSSION

SmNodC Shows Kinetic Properties Similar to ScCHS2—Full-length SmNodC was cloned and overexpressed in E. coli as a C-terminal mCherry fusion protein. Membrane fractions were
enzymes (21). Indeed, assay for the determination of shorter chito-oligosaccharide-A$_4$MU (Fig. 2). This novel assay is a fast and sensitive two-step mers on the reducing end by a chitinase to release fluorescent ence of 10 mM MgCl$_2$, whereas treatment with EDTA prevents /H11006-utilized during the reaction (linear range).

To further investigate the suitability of SmNodC as a model for fungal chitin synthases, we studied the susceptibility to the chitin synthase inhibitor Nikkomycin Z (Fig. 2E). Nikkomycin Z inhibits yeast chitin synthases in the 0.2–1000 $\mu$M range (27). Interestingly, Nikkomycin Z is a competitive inhibitor with the sub- strate UDP-GlcNAc, with a $K_i$ of 0.25 $\mu$M. This further highlights the suitability of SmNodC as a model for fungal chitin synthases, implying conservation of the active site in agreement with sequence alignments (Fig. 1). It further suggests that, together with the novel SmNodC assay, this system may offer new opportunities for the high throughput screen-based discovery of new CHS inhibitors.

The Topology of SmNodC Matches the CHS Core—Morgan et al. (5) have recently reported the first structure of a processive glycosyltransferase from the GT-2 family, BcsA, a bacterial cellulose synthase from Rhodobacter sphaeroides (Fig. 3A), suggesting a catalytic mechanism of cellulose synthesis and transport across the bacterial membrane. They further reported insights into the positioning and potential function of the CON1/CON2 motifs conserved across GT-2 family members and suggested key residues that are located in the active site and might be important for catalytic activity (5). We combined the BcsA crystal structure and sequence alignments (Fig. 1) to generate structural models of SmNodC and SccHS2 (Fig. 3A) to serve as a guide for the experimental verification of SmNodC topology and to probe the function of conserved residues by mutagenesis. Structural models of SmNodC and SccHS2 were generated with the RaptorX server (28), using the BcsA struc- ture as a guide. The SmNodC model was built with a 62% overall alignment score for the full-length protein and a 76% alignment score for the catalytic core (residues 46–284). The SmNodC structural model suggests three TM domains (Cys-4 to Lys-28, Leu-303 to Ile-329, and Trp-336 to Leu-360) and suggested key residues that are located in the active site and might be important for catalytic activity (5). We combined the BcsA crystal structure and sequence alignments (Fig. 1) to generate structural models of SmNodC and SccHS2 (Fig. 3A) to serve as a guide for the experimental verification of SmNodC topology and to probe the function of conserved residues by mutagenesis. Structural models of SmNodC and SccHS2 were generated with the RaptorX server (28), using the BcsA struc- ture as a guide. The SmNodC model was built with a 62% overall alignment score for the full-length protein and a 76% alignment score for the catalytic core (residues 46–284). The SmNodC structural model suggests three TM domains (Cys-4 to Lys-28, Leu-303 to Ile-329, and Trp-336 to Leu-360) and three cytoplasmic interface-leaning domains IF1 (187–202), IF2 (271–291), and IF3 (364–385) (Figs. 1 and 3A). The Topology of SmNodC Matches the CHS Core—Morgan et al. (5) have recently reported the first structure of a processive glycosyltransferase from the GT-2 family, BcsA, a bacterial cellulose synthase from Rhodobacter sphaeroides (Fig. 3A), suggesting a catalytic mechanism of cellulose synthesis and transport across the bacterial membrane. They further reported insights into the positioning and potential function of the CON1/CON2 motifs conserved across GT-2 family members and suggested key residues that are located in the active site and might be important for catalytic activity (5). We combined the BcsA crystal structure and sequence alignments (Fig. 1) to generate structural models of SmNodC and SccHS2 (Fig. 3A) to serve as a guide for the experimental verification of SmNodC topology and to probe the function of conserved residues by mutagenesis. Structural models of SmNodC and SccHS2 were generated with the RaptorX server (28), using the BcsA struc- ture as a template. The SmNodC model was built with a 62% overall alignment score for the full-length protein and a 76% alignment score for the catalytic core (residues 46–284). The SmNodC structural model suggests three TM domains (Cys-4 to Lys-28, Leu-303 to Ile-329, and Trp-336 to Leu-360) and three cytoplasmic interface-leaning domains IF1 (187–202), IF2 (271–291), and IF3 (364–385) (Figs. 1 and 3A).

To probe this predicted topology, we employed GFP and PhoA C-terminal fusion proteins to distinguish between the cytoplasmic and periplasmic regions of SmNodC. PhoA is only active when located in the periplasm, and GFP/ mCherry is known to only properly fold and, thus, fluoresce in the bacterial cytoplasm (23, 29). All constructs that do
have their C terminus in the cytoplasm will have a fluorescent GFP fusion (23) and will not show PhoA activity. Full-length (1–426) SmNodC expresses as a fluorescent mCherry fusion protein in E. coli, whereas a PhoA fusion does not possess any activity (Fig. 3B), suggesting that the C terminus is facing the cytoplasm, in agreement with the structural model (Fig. 3A). This is in contrast to an earlier study of NodC topology (22) where PhoA-fusion on a C-terminally truncated construct showed PhoA activity, which would indicate that the C terminus of NodC is located in the periplasm. We constructed a further 11 C-terminal SmNodC truncations, each with a C-terminal PhoA or GFP fusion. Fig. 3B shows all SmNodC-PhoA/GFP fusion proteins constructed and their fluorescence/PhoA activity. Twelve C-terminal fusion constructs were cloned, expressed, and investigated for their GFP fluorescence and PhoA activity. The numbers on the x-axis correspond to the C terminus of the construct. Signals were standardized to 100% for the highest signal, and fusion constructs with GFP fluorescence greater than 20% and a PhoA activity greater than 20% were considered positive results. SmNodC contains only a small loop between TM2 and TM3 that is located in the periplasm. The catalytic core (30–300) and the C-terminal domain (355–426) are clearly located in the cytoplasm. GFP fusion control protein FrdC contains a cytoplasmic C terminus, and PhoA control protein TarA has its C terminus located in the periplasm (23).

FIGURE 3. Structural model of SmNodC and ScCHS2 and topology mapping of SmNodC. A, structural model of SmNodC and ScCHS2. Shown is a schematic of BcsA (PDB code 4HG6, Ref. 5) and corresponding structural models calculated for SmNodC and ScCHS2. The membrane is indicated by two black lines. Structurally conserved TM and IF domains are labeled and colored as in Fig. 1. TM domains are colored in blue, and cytoplasmic interface leaning domains are colored in cyan. Non-conserved BcsA TM1–3 are shown in light blue. The structurally conserved N-terminal catalytic GT-A fold domain is shown with red α-helices, yellow β-strands, and green loops. B, topology mapping of SmNodC using GFP and PhoA fusions. Twelve C-terminal fusion constructs were cloned, expressed, and investigated for their GFP fluorescence and PhoA activity. The x-axis corresponds to the C terminus of the construct. Signals were standardized to 100% for the highest signal, and fusion constructs with GFP fluorescence greater than 20% and a PhoA activity greater than 20% were considered positive results. SmNodC contains only a small loop between TM2 and TM3 that is located in the periplasm. The catalytic core (30–300) and the C-terminal domain (355–426) are clearly located in the cytoplasm. GFP fusion control protein FrdC contains a cytoplasmic C terminus, and PhoA control protein TarA has its C terminus located in the periplasm (23).

Taken together, the experimental topology mapping approach (Fig. 3B) validates the SmNodC topology as derived from the structural model (Fig. 3A), showing that NodC enzymes contain three transmembrane domains that traverse the membrane in an out-in, in-out, out-in fashion, connected by a cytoplasmic hydrophilic core located between TM1 and TM2 and by a short periplasmic hydrophilic loop between TM2 and TM3 (Fig. 3A). The fourth domain is predicted to be a cytoplasmic interface leaning domain. This topological arrangement positions the catalytic domain into the cytoplasm, allowing the enzyme to access the cytoplasmic pool of the sugar nucleotide substrate UDP-GlcNAc. The Catalytic Machinery of SmNodC/ScCHS2 Consists of Three Conserved Motifs—the BscA structure was crystallized in complex with UDP and a cellulose product, facilitating the interpretation of the predicted active site in the SmNodC model. To probe the role of active site residues, we designed

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specific point mutants and tested the effects on chitooligosaccharide synthesis. According to the sequence alignment (Fig. 1), SmNodC Phe-58 corresponds to Tyr-148 in cellulose synthase and appears to form a hydrophobic stacking interaction with the UDP-GlcNAc uracil moiety (Fig. 4A). In agreement with this, the F58A mutation inactivates SmNodC (Fig. 4B), similar to the equivalent mutation (Y298A) in ScCHS2 (8). The structural model further shows that the side chain of SmNodC Asp-140 (Asp-441 in ScCHS2) is in proximity to the side chain of SmNodC Asp-241 (Asp-562 in ScCHS2), coordinating the Mg\(^{2+}\) ion that is essential for catalysis (Fig. 4A). Mutation of either of these residues to alanine or asparagine inactivate the enzyme (Fig. 4B), similar to equivalent mutations of these residues in ScCHS2 (8).

The conserved Q(Q/R)XRW CON1 motif, characteristic of processive GT-2 enzymes, forms an \(\alpha\)-helix lining the active site (Fig. 4A). Mutations of any of these residues to an alanine or asparagine inactivate SmNodC (Fig. 4B), similar to the corresponding ScCHS2 mutations (8). The model suggests that the invariant residues Q(Q/R)XRW are predicted to face the active site, whereas the invariant Q(Q/R)XRW residues are positioned on the back of this helix (Fig. 4A). Trp-281 of SmNodC (Trp-605 in ScCHS2) is potentially involved in \(\pi-\pi\) stacking interactions with the GlcNAc sugar in the +2 subsite (Fig. 4A). This is in agreement with kinetic data showing that mutation of W281A (Trp-605 in ScCHS2) abolishes catalytic activity (Fig. 4B). Similarly, the side chain of Arg-280 (Arg-604 in ScCHS2) is predicted to bind the negatively charged diphosphate moiety of UDP-GlcNAc (Fig. 4A), consistent with the observation that the alanine mutants of this residue in SmNodC (Fig. 4B) and ScCHS2 are inactive (8).

According to the reaction mechanism proposed for processive, inverting glycosyltransferases, two side chain carboxylates are required (30). These have been proposed to act as the general base, abstracting the proton of the acceptor substrate, and the second carboxylate assists to coordinate the leaving group (Mg\(^{2+}\)) departure (30) (SmNodC Asp-140). It is not known precisely how the catalytic base is regenerated (deprotonated), but this presumably, ultimately, involves the transfer of a proton to a water molecule.
NodC and class II CHS share a conserved EDR motif (Fig. 1, SmNodC 240–242 and ScCHS2 561–563). The SmNodC model reveals that the side chain of Asp-241 is positioned ~4.0 Å from the hydroxyl group of the acceptor sugar (Fig. 4A). This aspartate may therefore act as the catalytic base, activating the sugar acceptor for nucleophilic attack. Glu-240 and Tyr-214 are in proximity to Asp-241, perhaps tuning its pKₐ (Fig. 4A). The mutation of any of these conserved residues inactivates SmNodC and ScCHS2 (Fig. 4B) (8). Mutation of Arg-215, which is seen to position Tyr-214 in the SmNodC model, reduces the relative catalytic activity to ~40%. With the help of the structural model, we propose that the side chain of this conserved arginine is important, but not essential, for the structural integrity of the GT-A folded catalytic domain. Mutation of the final residue of the EDR motif, Arg-242 in SmNodC (Arg-563 in ScCHS2), and mutation of Leu-244 in SmNodC (Leu-565 in ScCHS2) inactivate the enzyme (Fig. 4B) (8). The SmNodC model suggests that these residues may be required for precise positioning of the α-helix with the catalytic EDR motif (Fig. 4A).

Two control mutations away from the active site, Ser506A and Ser174A (surface-exposed, minor reductions in activity, Fig. 4B) did not affect the catalytic activity.

The SmNodC Active Site Forms a Molecular Ruler—One of the key differences between SmNodC and ScCHS2/BcsA is that SmNodC synthesizes only short, soluble chitooligosaccharides, up to (GlcNAc)₅, whereas NodC synthesizes only short, soluble chitooligosaccharides, SmS174A (surface-exposed, minor reductions in activity, Fig. 4B) (8). This reveals that the side chain of Asp-241 is positioned until the C terminus indeed forms the product-binding site (Figs. 3A and 4A). The SmNodC model reveals that the C-terminal domain (Ile-262 to Ser506A in SmNodC) regulates chitooligosaccharide product length (31). The NodC model reveals that both enzymes share a very similar nucleotidyltransferase mechanism, resulting in the next 4-hydroxyl acceptor approaching the catalytic center from the opposite site than the acceptor in the previous step (5). The mechanism proposed here, sugars translocating from the +1 into +2 subsite would rotate every second subsequent step, whereas sugars translocating into the +3/+4 subsites could remain of a fixed orientation, requiring these subsites to accommodate the N-acetyl moieties in both sugar conformations. Energy for translocation/rotation may be supplied as steric strain on the +1 sugar introduced with each processive glycosyltransferase step.

Mechanism of Chitin Synthesis and Transport—We identified catalytic residues by structural modeling and mutagenesis. It is possible to predict the SmNodC product-binding subsite (+1 to +5) of the acceptor polysaccharide by superimposing the glucose polymer from the BcsA structure onto the SmNodC model (Figs. 4A and Fig. 5). NodC enzymes synthesize polysaccharides of up to chitin pentaoase, whereas ScCHS2 and BcsA synthesize long polysaccharides. The main product of SmNodC is chitotetraose (31). The product-binding tunnel reveals distinct binding sites that accommodate the N-acetyl moiety of the chitoooligosaccharide (Fig. 5). Together with the data on the catalytic residues, this allows us to propose a reaction mechanism for processive chitoooligosaccharide synthesis. The acceptor GlcNAc moiety sits in the +1 site with the 4-hydroxyl group pointing toward the UDP-GlcNAc binding site (Fig. 6). The acceptor sugar is activated by the catalytic base (Asp-241 in SmNodC) and performs a nucleophilic attack on the anomeric carbon of UDP-GlcNAc, generating a β-(1,4)-glycoside. Upon completion of the transfer reaction, UDP leaves the active site. At the same time the +1 sugar moves into the +2 pocket, whereas the newly added GlcNAc moves and rotates into the +1 site. This produces an acceptor sugar position/confirmation that is identical to the first step. An alternative is the mechanism proposed for BscA, which does not involve sugar rotation, resulting in the next 4-hydroxyl acceptor approaching the catalytic center from the opposite site than the acceptor in the previous step (5).

CONCLUSIONS

SmNodC was overexpressed in E. coli cells as an active mCherry–His tag fusion protein. We developed a novel, efficient, high throughput-compatible, non-radioactive assay that allowed us to determine Michaelis–Menten kinetics and metal dependence. With the help of this assay, we were able to show that the CHS inhibitor Nikkomycin Z is a potent competitive NodC inhibitor. Therefore, we propose that SmNodC is a useful model to screen for small molecule inhibitors and to identify novel molecules that might also inhibit the fungal chitin synthases. Structural insights into the active site of SmNodC and comparing conserved active site residues between SmNodC and ScCHS2 reveals that both enzymes share a very similar nucleo-
tide sugar- and acceptor-binding site. The topological arrangement was validated by biochemical experiments and revealed that NodC enzymes contain three membrane-spanning and three cytoplasmic interface-leaning domains, similar to the recently determined structure of a cellulose synthase (5), and the predicted CHS structure. Furthermore, our topology studies correlate with eukaryotic GT-2 enzymes (5, 32).

ScCHS2 contains six TM domains, four of which can be structurally modeled on the basis of the bacterial cellulose synthase structure (Fig. 3A). The remaining two TM domains are predicted to be part of a chitin transport channel that is absent from SmNodC because this enzyme only synthesizes short chitooligosaccharides for further processing in the cytoplasm.

Amino acids conserved between SmNodC and CHS were targeted by mutagenesis. The SmNodC mutants show inactivation, similar to the corresponding ScCHS2 mutants. On the basis of these data, we propose a reaction mechanism where the only sugar that rotates is the +1 sugar because it translocates to the +2 subsite, and only every second step. This preserves the up/down arrangement of the N-acetyl groups as seen in crystalline chitin and consistently presents the 4-OH hydroxyl of the growing acceptor to the active site in the same orientation.

NodC enzymes lack the transmembrane domains that are present in BcsA and CHS2 to form a product translocation channel across the lipid bilayer. Our structural model further revealed that the product-binding site of SmNodC is defined by two residues that are conserved among NodC enzymes. These define a molecular ruler to synthesize chitooligosaccharides of limited length, whereas CHS encode a chitin transporter channel presumably built from at least four TM domains.

We propose SmNodC as a model system to study chitin synthases on a molecular and structural level to elucidate the reaction mechanism on a structural level of chitooligosaccharide synthases and to identify novel CHS inhibitors because we propose that novel inhibitors of NodC enzymes will be interesting lead compounds to inhibit chitin synthases.

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