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Probing the mechanism of a fungal glycosyltransferase essential for cell wall biosynthesis. UDP-Chitobiose is not a substrate for chitin synthase †

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Chitin synthase is responsible for the biosynthesis of chitin, an essential component of the fungal cell wall. There is a long-standing question as to whether "processive" transferases such as chitin synthase operate in the same manner as non-processive transferases. The question arises from analysis of the polysaccharide structure – in chitin, for instance, each sugar residue is rotated ≈180° relative to the preceding sugar in the chain. This requires that the enzyme account for the alternating "up/down" configuration during biosynthesis. An enzyme with a single active site, analogous to the non-processive transferases – would have to accommodate a distorted glycosidic linkage at every other synthetic step. An alternative proposal is that the enzyme might assemble the disaccharide donor, addressing the "up/down" conformational problem prior to polymer synthesis. We present compelling evidence that this latter hypothesis is incorrect.

We wish to report our recent efforts to clarify an important aspect of the mechanism of chitin synthase, the processive glycosyltransferase responsible for the polymerization of uridinediphosphoryl-*N*-acetylglucosamine (UDP-GlcNAc) to form chitin (poly-*β*-1,4-GlcNAc).[1,2] Chitin is a rigid rod-like oligosaccharide, and an essential component of the fungal cell wall. As it is absent in mammals, chitin synthase (CS) has long been regarded as a promising target for antifungal therapeutics. Among the challenges in developing inhibitors of CS are the dearth of inhibitory natural products and the ubiquity of UDP-GlcNAc-dependent transferases.[3] With regard to the latter issue, there would be clear value in identifying differences between CS and the numerous other *N*-acetylglucosaminyl transferases, as this would allow for the selective targeting of this important enzyme.

There is a long-standing question as to whether "processive" transferases such as chitin synthase, which synthesize polymeric oligosaccharides without releasing synthetic intermediates, operate in the same manner as the comparatively well understood non-processive transferases, which transfer a single activated sugar to an acceptor substrate and then release the product.[4–6] This question arises from the conformation of the extended structure of polysaccharides. In chitin, a representative case, each residue is ≈180° out of phase with the preceding sugar in the polymer chain (Fig. 1), and the enzyme must somehow account for this alternating "up/down" configuration during biosynthesis. If processive and non-processive transferases share a common mechanism – addition of a monosaccharide donor (UDP-*N*-acetylglucosamine, UDP-GlcNAc) to the non-reducing end of the growing polymer – then every other synthetic step must lead to a distorted intermediate (Fig. 2, path a).[7,8] While this mechanism cannot be excluded, there is clear incentive for the consideration of other mechanisms. One alternative proposal is that the enzyme or an associated protein preassembles the disaccharide donor (UDP-chitobiose), allowing extension by two residues at a time and resolving the "up/down" conformational issue prior to polymer synthesis (Fig. 2, path b).[6b–d] We describe here the synthesis of UDP-chitobiose, the intermediate required by the disaccharide preassembly hypothesis, and show that it is not a kinetically competent substrate for chitin synthase.

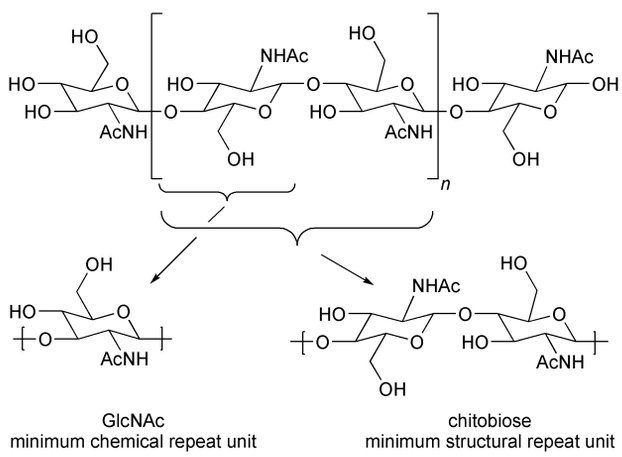
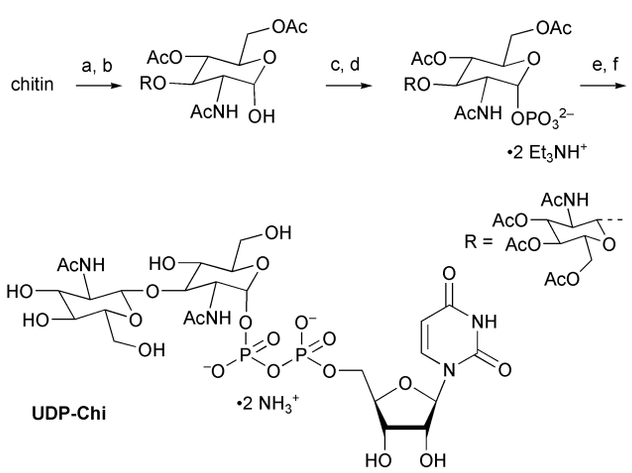


Fig. 1

Synthesis of UDP-chitobiose (UDP-Chi) began with a modified procedure for the degradation of chitin in hot AcOH–H_2SO_4, which consistently provided a *ca.* 10% yield of peracylated chitobiose without chromatography (Scheme 1).[9,10] Similar



Scheme 1 a) H_2SO_4, Ac_2O, △, ≈10%; b) N_2H_4·HOAc, DMF, 83%; c) i. LDA, THF ii. $[(BnO)_2PO]_2O$, 77%; d) H_2, Pd/C, CH_3OH–NEt_3, 99%; e) UMP morpholidate, tetrazole, py, 55%; f) CH_3OH–NH_3, 85%.

† Electronic supplementary information (ESI) available: A. General experimental details; B. Synthesis of UDP-chitobiose (UDP-Chi); C. Procedure for chitin synthase assay; D. Data from UDP-Chi assays and E. K_i. determination. See http://www.rsc.org/suppdata/ob/b2/b208953j/

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Table 1 Evaluation of UDP-Chi as a chitin synthase substrate

	UDP-GlcNAc	UDP-Chi	^{3}H-UDP-GlcNAc	^{3}H-UDP-Chi[a]	Relative activity[a]
1	0.75 mM	—	+	—	100 %
2	—	0.75 mM	—	+	≤2%
3	—	3.75 mM	—	+	≤2%
4	—	—	—	+	≤2%
5	0.75 mM	0.75 mM	+	—	83%
6	—	0.75 mM	+	—	≤2%
7	—	—	+	—	≤2%

[a] [^{3}H-substrate]≈0.01 mM.

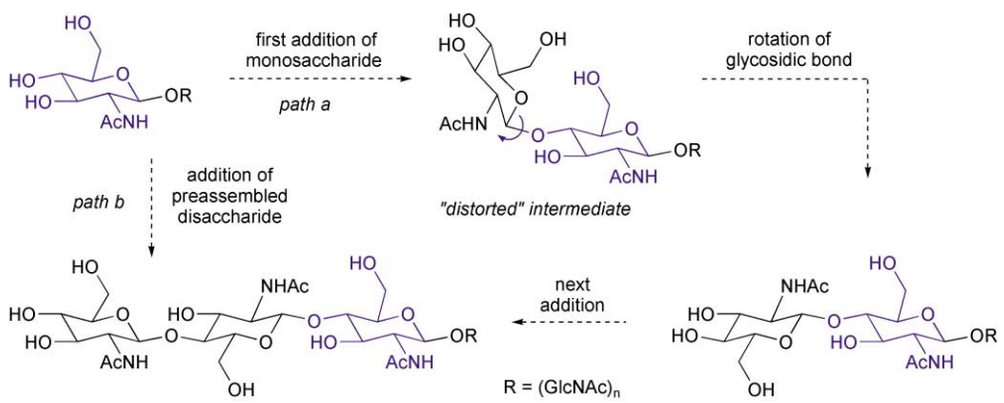
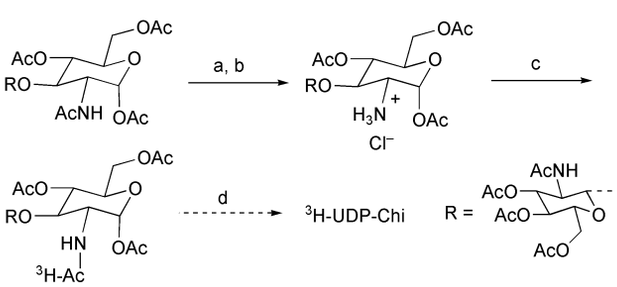


Fig. 2

to peracylated GlcNAc, the anomeric acetate of peracetyl-chitobiose could be selectively removed by treatment with hydrazine acetate.[9,11] Subsequent phosphorylation with tetrabenzyl pyrophosphate and removal of the benzyl groups afforded the corresponding anomeric phosphate, isolated as the ammonium salt.[12] Coupling of this phosphate with the morpholidate of UMP,[13] followed by deacylation, afforded UDP-Chi in 30% yield from peracetyl chitobiose.

Chitin synthase assays rely on the use of radiolabelled nucleotide sugar substrates, which necessitated the synthesis of ^{3}H-UDP-Chi (Scheme 2). The acetamide of the reducing sugar



Scheme 2 a) HCl, AcOH–Ac_2O, 61%; b) 1 eq. H_2O, acetone, 72%; c) AcO–^{3}H-Ac_2O, py, 83%; d) as Scheme 1.

was selectively deacylated by conversion to the oxazoline, *via* the anomeric chloride, followed by selective hydrolysis of the oxazoline to afford the anomeric acetate with an ammonium group at C2.[14] Acylation with Ac_2O–^{3}H-Ac_2O provided tritiated peracetyl chitobiose in 37% overall yield, which was converted to the UDP derivative by the preceding method.

Chitin synthase activity was assayed with membrane preparations from yeast (*S. cerevisiae*) by the procedure of Orlean.[15] Reactions were run for 1 hour, at which time the protein was denatured and the chitin precipitated by the addition of cold trichloroacetic acid. Assay mixtures were then filtered and rinsed to isolate precipitated chitin, and total incorporated radioactivity was measured by scintillation counting. "100% activity" was typically 12–13,000 cpm h^{-1}, with a time-independent background of 250–300 cpm; the background radioactivity for high-concentration ^{3}H-UDP-Chi assays was slightly higher due to higher initial radioactivity (Table 1). All data are the average of 2 or more experimental trials.

Initial evaluation revealed that UDP-Chi is not a viable substrate for chitin synthase: even at elevated [UDP-Chi], no radioactivity incorporation above background was observed (Table 1, entries 1–4). Control experiments ruled out the possibility that UDP-Chi was a viable substrate but required the presence of UDP-GlcNAc as a co-substrate or polymerization initiator (Table 1, entries 5–7).[16] Further, measurement chitin formation at fixed [UDP-GlcNAc] with variable [UDP-Chi] indicated that UDP-Chi is an inhibitor of chitin synthase, with K_i = 3.3 mM (IC_{50} = 10.0 mM).[17,18] This is similar to the K_M for UDP-GlcNAc (0.5 mM).[15] Measurement of chitin formation with [UDP-Chi] = 7.5 mM and variable [UDP-GlcNAc] demonstrated that addition of sufficient UDP-GlcNAc (≥15 mM) leads to full recovery of enzymatic activity, which indicated that the inhibition is competitive.[19] These data support the idea that UDP-GlcNAc and UDP-Chi bind to a common site in chitin synthase, and that the failure of the enzyme to convert UDP-Chi to chitin truly reflects lack of reactivity rather than lack of binding affinity.

These experiments thus reasonably exclude the involvement of UDP-Chi as a transient intermediate in chitin biosynthesis. Given the homology among processive glycosyltransferases, it is likely that this conclusion can be extended to other enzymes, such as cellulose synthase.[20] With this mechanism excluded, there remain two limiting cases to consider: first, as noted, that chitin synthase has a single active site and somehow compensates for the formation of a high-energy intermediate required by every other synthetic step; second, that chitin synthase in fact has two active sites, one for each orientation of the GlcNAc electrophile. Efforts are underway to test this latter hypothesis.

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Dr Herman van Halbeek, Mr Steven Adams and Mr Dennis Koehler (UCSD) for technical assistance, and Professors Scott Singleton (Rice University) and Jack Kyte (UCSD) for helpful discussions.

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17 K_i values were obtained from the relationship $K_i = IC_{50}/(1+[\text{substrate}]/K_m)$ (ref. 18). Assays were performed at [UDP-GlcNAc] = 1.0 mM. K_M = 0.5 mM, so this reduces to $K_i = IC_{50}/3$. IC_{50} was determined by non-linear least-squares fitting of a plot of inhibition (%) *vs.* log [UDP-Chi] (measured at [UDP-GlcNAc] = 1 mM. using the Prism3 software package (Graphpad Inc., San Diego, CA); log IC_{50} for UDP-Chi was determined to be 1.0 ± 0.1 (95% confidence limits), providing $IC_{50} = 10.0 +2.6/-2.1$ (7.9 − 12.6).

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