Chitin biosynthesis during *Blastocladiella* zoospore germination: Evidence that the hexosamine biosynthetic pathway is post-translationally activated during cell differentiation*

(post-translational regulation/end product inhibition/cell-wall formation)

C. P. SELITRENNIKOFF, D. ALLIN, AND D. R. SONNEBORN

Department of Zoology, University of Wisconsin, Madison, Wis. 53706

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ABSTRACT  
*De novo* construction of a chitinous cell wall accompanies *Blastocladiella emersonii* zoospore germination. At least an order of magnitude increase in total hexosamine occurs during germination. This increase is into polymer (chitin) and occurs on schedule in the presence of cycloheximide. Uridine-5'-diphospho-N-acetylglucosamine (UDPGlcNAc), both the end product of hexosamine biosynthesis and a substrate for chitin biosynthesis, is a potent inhibitor of the activity of the first pathway-specific enzyme of hexosamine biosynthesis in zoospore extracts. Certain uridine nucleotides, not perceptibly influencing the activity of the first enzyme per se, counteract the inhibitory effects of UDPGlcNAc. The concentration of UDPGlcNAc in the zoospore is sufficient to act as an inhibitor of the enzyme, but the amount of UDPGlcNAc is insufficient, by an order of magnitude, to account for the chitin synthesized during germination. Both the production of UDPGlcNAc and its utilization for chitin synthesis appear to be post-translationally regulated in zoospores and during zoospore germination.

During the germination phase of the life cycle of the water mold *Blastocladiella emersonii* the nonmotile zoospores rapidly convert to a sessile cell capable of vegetative growth. Accompanying this conversion is the *de novo* construction of a cell wall whose major component by weight is chitin. Morphologically, the cell wall appears on schedule in the presence of cycloheximide at protein-synthesis-inhibiting levels (2–4), suggesting that whatever the controls over initial wall formation are, they do not include a requirement for concomitant protein synthesis.

The terminal enzyme in chitin biosynthesis, chitin synthetase, is present at high specific activity in disrupted zoospore preparations (5, 6) and appears to be preferentially localized in vesicles morphologically characteristic of the zoospore cell type (6). Vesicles fused with the plasma membrane can be observed during the period of initial cell wall formation (7). As previously hypothesized (2, 6, 7), such vesicle–plasma membrane fusions may trigger the construction of the cell wall.

Information presented in this paper indicates that this hypothesis does not furnish a sufficient explanation for the abrupt production of chitin during germination. In particular, the total hexosamine content of the zoospore is found to be insufficient, by at least an order of magnitude, to account for the chitin present after the cell wall is formed. Enzyme activities corresponding to each of the four pathway-specific reactions of hexosamine biosynthesis have now been found in zoospore extracts (6, 8, 9, 1). Thus, while the enzymatic machinery to provide substrates for the chitin synthetase reaction is present in the zoospore, the zoospore does not in fact contain sufficient substrates. We report here that: (a) cycloheximide does not inhibit the initial, abrupt production of chitin during germination; (b) uridine-5'-diphospho-N-acetylglucosamine (UDPGlcNAc), a substrate for the synthetase reaction, but also the end product of hexosamine biosynthesis, is a potent negative regulator of the first pathway-specific enzyme of the hexosamine biosynthetic pathway; (c) the concentration of UDPGlcNAc in the zoospore is sufficient to act as a feedback regulator of this first enzyme; and (d) certain uridine nucleotides, not perceptibly influencing the activity of the first enzyme per se, counteract the inhibitory effects of UDPGlcNAc.

MATERIALS AND METHODS

Stock cultures were maintained on standard Cantino peptone–yeast–glucose (PYG) agar and subcultured daily on a 24 hr, 20°C life cycle schedule. Freshly released zoospores were harvested by flooding the surfaces of agar cultures with distilled water. Zoospores for some experiments involving hexosamine measurements were derived from liquid cultures grown and sporulated under conditions similar to those previously described (10); the measurements appeared not to be affected by the source of zoospores. Harvested zoospores were immediately filtered through Whatman 541

Abbreviations: UDPGlcNAc, uridine-5'-diphospho-N-acetylglucosamine; GlcNAc, N-acetylglucosamine; GlcN-6-P, glucosamine 6-phosphate; Fru-6-P, fructose 6-phosphate; CUMP, uridine-5'-3'- cyclic monophosphate; PYG, peptone–yeast–glucose; GS, germination solution.

* This paper is dedicated to Prof. T. M. Sonneborn and is to be included as part of a Festschrift in his honor. Twenty-seven years ago, he published a provocative review article entitled "Beyond the Gene" (1). Near the beginning of the article, he wrote: "As has often happened in the history of science, after a general principle is established, exceptions are reported one by one; they are then ignored or assimilated as well as possible into the current pattern of thought until accommodation is so difficult that a revision of the principle is undertaken. ... In the same article, Sonneborn attempted to relate the available findings from his laboratory to "implications for developmental differentiation." If there is a thread connecting the present article to Sonneborn's "current pattern of thought," it is with respect to this topic. The general principle is the "differential protein synthesis hypothesis." We are aware of no one, including ourselves, who seriously doubts the widespread importance of the principle. However, we report here on what we believe to be an exception to the sufficiency of that principle. We attempt no general revision of principle. We do wish to continue to stress, however, that the twin general problems of spatial and temporal localization, which lie at the heart of "developmental differentiation," appear to demand additional "options" than just the acts of transcription and translation per se. With this sentiment, we know we are in agreement with Prof. Sonneborn.

† C. P. Selitrennikoff and D. R. Sonneborn, manuscripts submitted.
filter paper to remove sporangial ghosts and cell types other than zoospores. The filtered suspensions were washed with cold distilled water by centrifugation (500 × g, 5 min) and the washed pellets were either frozen, lyophilized, and stored in a dessicator at −20° (for enzyme assays and for determination of hexosamine content of zoospores) or diluted into spinner flasks containing germination solution (GS: 50 mM KCl, 10 mM MgCl₂, 1 mM CaCl₂, 1 mM Tris-maleate buffer, pH 6.7) for experiments involving measurements of total hexosamine content during germination. At selected times during germination, samples were withdrawn from the spinner flasks for scoring cell types (7) and for measurement of total hexosamine content. The latter samples were immediately poured into centrifuge bottles containing ice, pelleted, washed once with cold distilled water, and lyophilized. In experiments where samples were harvested at 5 min intervals, the samples were harvested into cold 10% trichloroacetic acid (final concentration), incubated overnight in the cold, centrifuged, and evenly suspended by sonication in a small volume of distilled water.

Total hexosamine was measured on weighed samples (≥10 mg for zoospores; ≥2 mg for samples harvested during germination) that were hydrolyzed in 4 M HCl at 110° in vacuum-sealed tubes for 30 min to 10 hr. The hydrolyzed samples were evaporated to dryness over NaOH and dissolved in 0.01 M HCl. Hexosamine was measured by the procedure of Reissig et al. (11). Blanks lacking acetic anhydride were used for each hydrolysate to control for color arising during hydrolysis. For many experiments, crude hydrolysates were applied in 100- to 200-μl volumes to small (about 0.3 ml) Dowex-50 (H⁺) preparative columns in disposable pasteur pipettes plugged with glass wool. The columns were washed with 10 column volumes of distilled water and then hexosamines were eluted with 10 column volumes of 0.5 M HCl. The eluates were concentrated, redissolved, and measured as above for crude hydrolysates. Recoveries from the columns, both of authentic glucosamine and of total hexosamine in samples, were >98%. The hexosamines in preparative column eluates from zoospores were further identified by analytical chromatography on a previously calibrated Dowex-50 (H⁺) column (1 × 10 cm) as described by Wheat (12), with the exception that the column was developed with 0.2 M HCl rather than 0.3 M HCl. The measurable hexosamine content eluted as a single peak with an Rf identical to authentic glucosamine and clearly separate from other hexosamines. Overall recovery through both the preparative and analytical columns was about 90%. The types of hexosamine compounds in unhydrolyzed zoospores were examined after quantitative extraction of total hexosamine by the hot water method of Rothman and Cabib (13). The extracts were lyophilized, dissolved in distilled water, and chromatographed on a Dowex-1 (formate) column (1 × 9.7 cm) as described by Molnar et al. (14). A linear gradient of ammonium formate (0–0.75 M, pH 7.5; 400 ml total eluate collected in 4-ml fractions) was used.

For enzyme assays, 10 mg of lyophilized zoospores were suspended in 1 ml of 100 mM phosphate buffer, pH 6.8, containing 600 mM sucrose, 1 mM KCl, and 1 mM NaN₃ EDTA (buffer A) by pipetting up and down several times with a pasteur pipette. This procedure resulted in complete lysis of zoospores but not of other minor contaminating cell types. The lysates were centrifuged at 10,000 × g for 20 min in the cold. To separate small molecules from enzyme activity, the supernatants were applied to 1 × 10.5 cm Sephadex G-25 columns previously equilibrated with buffer A and developed with the same buffer. The first two 750-μl fractions after the void volume were retained for enzyme assays; these fractions contained about 50% of the total enzyme activity and were virtually free (<1%) of added radioactive glutamine, fructose 6-phosphate (Fru-6-P), and UDPGlcNAc. For experiments examining the effects of UDPGlcNAc on enzyme activity, buffer B (addition of 10 mM glutamine to buffer A and readjustment to pH 6.8) was used from the beginning of the procedure, as enzyme activity was partially desensitized to inhibition by UDPGlcNAc when zoospore extracts were prepared in the absence of glutamine. 1-L-Glutamine:D-fructose-6-phosphate amidotransferase [formerly EC 2.6.1.16; transferred to EC 5.3.1.19, 2-amino-2-deoxy-D-glucose-6-phosphate ketol-isomerase (amino-transferring)] activity was assayed in 150-μl reaction mixtures containing various concentrations of substrates and effectors; 50 μl of the above combined column fractions were added to initiate the reactions. The formation of glucosamine 6-phosphate (GlcN-6-P) was determined by the procedure of Ghosh and Roseman (15), with slight modifications as described in detail elsewhere. Each point in the figures is the average of two determinations (<10% difference between readings). Protein concentration was determined by the method of Lowry et al. (16), using bovine serum albumin (fraction V) as a standard. A unit of enzyme activity is the quantity which directed the formation of 1 nmol of GlcN-6-P per min at 25°. Michaelis–Menten constants (Km) were determined by the standard Lineweaver–Burk graphic method; the inhibition constants (Ki) were determined graphically by the method of Dixon (17).

**RESULTS**

**Hexosamine Content of Zoospores versus Germinating Cells.** Measurements of the total hexosamine of zoospores yielded values of 0.05 ± 0.01% (standard deviation) by weight prior to acid hydrolysis and 0.16 ± 0.04% after hydrolysis (eight independent determinations). Maximal values were achieved after mild hydrolysis (1 M HCl, 110°, 30 min), much milder than required to hydrolyze the glycosidic bonds in polymerized hexosamine (Fig. 1 and below). The hexosamine content of zoospores could be quantitatively extracted by the hot water method of Rothman and Cabib.
The spores. With zoospores UDPGlcNAc osamine (after in our UDPGlcNAc intracellular concentration was calculated to be $6.3 \times 10^{-4}$ M).

(13), i.e., measurements of total hexosamine (after acid hydrolysis) in unextracted zoospores and hot water extracts were indistinguishable. About 70% of the extracted hexosamine content consistently cochromatographed with authentic UDPGlcNAc (Fig. 2) and gave a positive reaction in the Reissig et al. (11) hexosamine assay only after mild hydrolysis (1 M HCl, 30 min). This material is UDPGlcNAc, rather than other UDP hexosamines, since only one hexosamine peak was detected by analytical Dowex-50 (H+) chromatography of hydrolyzed zoospore extracts (recovery ≥90%) and the peak had an $R_F$ identical to authentic glucosamine (see Materials and Methods). From three independent quantitations of material cochromatographing with authentic UDPGlcNAc, we estimate the intracellular concentration of UDPGlcNAc to be in the range 1.7 to $6.3 \times 10^{-4}$ M (‡, Fig. 2 legend).

Upon germination, a rapid increase in total hexosamine was observed; by 20 min of germination, a value of 1.6 ± 0.3% by weight (five determinations) was observed, and by 30 min, a value of 2.5 ± 0.35% (six determinations) was observed. The increased hexosamine content resides virtually entirely in polymerized hexosamine since: (a) strong hydrolysis (4 M HCl; 4–8 hr) was required to release the increased levels of hexosamine (Fig. 1); and (b) virtually all of the increased hexosamine was recovered in low-speed (500 $\times$ g) pellets of sonicated samples (cell-wall-containing fraction) and was KOSO-insoluble (chitin). When germination was initiated in the presence of cycloheximide levels known to reversibly inhibit protein synthesis (2, 3), the initial burst in polymerized hexosamine content was observed (Figs. 1 and 3). In several experiments, cycloheximide did appear to inhibit the continued accumulation of polymer beyond a level of about 2% by weight (see, for example, Fig. 3B).

Characterization of Amidotransferase Activity in Zoospore Extracts. Under the conditions of assay for the amidotransferase, the formation of GlcN-6-P by crude extracts of zoospores was linear with respect to time (through 20 min) and protein concentration (21-81 μg). The reaction was specific with respect to both Fru-6-P and glutamine; neither glucose-6-P nor galactose-6-P effectively substituted for fructose-6-P and asparagine did not substitute for glutamine. The effects of various concentrations of Fru-6-P and glutamine on enzyme reaction velocity are shown in Fig. 4. From these data, the $K_m$ of the crude enzyme for Fru-6-P was calculated to be about $1.6 \times 10^{-3}$ M and for glutamine to be about $1 \times 10^{-3}$ M. Even though estimated with crude enzyme, these values are similar to those found with partially purified amidotransferases from several sources.

B. emersonii zoospore amidotransferase activity is strongly inhibited by UDPGlcNAc. The data in Fig. 5 show that UDPGlcNAc behaves kinetically as a competitive inhibitor [nomenclature of Cleland (25)] with respect to Fru-6-P (calculated $K_i$, about $5 \times 10^{-8}$ M) and as an uncompetitive inhibitor with respect to glutamine (calculated $K_i$, about $7 \times 10^{-5}$ M). Other UDP sugars were found to be strikingly less inhibitory than UDPGlcNAc (e.g., at saturating substrate concentrations, 0.1 mM UDPGlcNAc, UDPglucose, or UDPgalactose inhibited amidotransferase activity by 72%, 20%, or 10%, respectively). The inhibition of the amidotransferase activity by UDPGlcNAc is likely to be due to its formation from UDPgalactose by the action of the UDPgalactose-4-epimerase present in B. emersonii.
The effects of uridine nucleotides on zoospore amidotransferase activity were also determined. Under conditions where both substrates were saturating, UTP, UDP, UMP, and 3'5'-cyclic UMP (cUMP) at concentrations varying from 1 to 5 mM had less than 4% effect on the enzyme reaction (data not shown). However, UTP and UDP had marked stimulatory effects on the UDPGlcNAc-inhibited reaction; the effects of UMP and cUMP were comparatively slight (Fig. 6). These results are similar to those of Winterburn and Phelps (26) using partially purified amidotransferase from rat liver.

**DISCUSSION**

During the short period of initial cell-wall formation accompanying *B. emersonii* zoospore germination, the total hexosamine level increases at least an order of magnitude; virtually all of the increase is into polymer (chitin). These results indicate that: (a) the zoospore does not contain sufficient hexosamine substrates for the chitin synthesized during germination, and (b) the block to chitin synthesis is abruptly released during germination. We consider these two points below.

With regard to the first point, we propose that the hexosamine biosynthetic pathway is end-product-inhibited in the zoospore. While high specific activities for each of the four enzymes of the pathway have been found in zoospore extracts (6, 8, 9, 1), the end product (UDPGlcNAc) inhibits the activity of only the first enzyme.\(^1\) Inhibition of amidotransferase activity by UDPGlcNAc (Fig. 5, A) has been observed with enzyme preparations from several other eukaryotes, but not from bacteria.\(^2\) The concentration of UDPGlcNAc in the zoospore (see Results and \(^2\)) appears to be at least equivalent to that which yields maximal inhibition by UDPGlcNAc of *in vitro* amidotransferase activity (Fig. 5).

Since UDPGlcNAc concentration is also in the range of the apparent \(K_m\) of chitin synthetase for diacetylgalactosamine formation (5), how is it that the zoospore does not consume the available UDPGlcNAc in the chitin synthetase reaction? Two different, though not mutually exclusive, answers can be offered: (a) as mentioned above, there is evidence that chitin synthetase is "packaged" in the zoospore in membrane-bound particles; perhaps in *vivo* substrates are inaccessible to the enzyme within these particles or the enzyme within the particles is in an inactive form (there is precedent for the latter alternative; see ref. 28); (b) not only chitin but also \(N\)-acetylglucosamine (GlcNAc) may be limiting for chitin synthetase activity in the zoospore. Even if all the hexosamine recovered in fraction A, Fig. 2, were GlcNAc (all neutral or positively charged hexosamine derivatives would be recovered in this fraction; see ref. 14), the estimated intracellular concentration would be on an order of magnitude less than the reported (5) apparent \(K_m\) of chitin synthetase for GlcNAc (see, however, \(^3\)).

It is most unlikely that the abrupt release of the block to chitin synthesis during germination is controlled in any way by the sudden synthesis of one or more enzymes in the path-

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\(^1\) While this paper was being written, \(K_m\) and \(K_i\) values for *Blastocladiella* zoospore amidotransferase activity were published (27). The reported values are similar to those reported here and are compared in a manuscript submitted.

\(^2\) If chitin is present in zoospores, it constitutes \(\leq 0.01\%\) by weight. A lower value could not be assigned in the present work because the filtered zoospore preparations were contaminated with 0.1-0.5% germinating cells, as judged by the sensitivity of the cell populations to lysis by detergent [zoospores, but not cell wall-containing, cells are sensitive to detergent lysis (7)].
way to chitin synthesis. Not only have high specific activities for all five pathway-specific enzymes been found in zoospore extracts, but also both cell-wall formation (2, 3) and initial chitin production (this paper) occur on schedule in the presence of cycloheximide. Part of the release mechanism may indeed involve activation of the chitin synthetase reaction upon fusion of vesicles with the plasma membrane. Vesicle-plasma membrane fusion as a means of bringing molecules involved in cell-wall construction to the cell surface appears to be a general phenomenon; documentations have been widely reported using a variety of eukaryotic microorganisms and higher plants. Nevertheless, activation of the chitin synthetase reaction per se does not solve the problem of providing an order of magnitude increase in total hexosamine. The flux of metabolites in the pathway to UDPGlcNAc must undergo an abrupt, radical increase. This part of the release mechanism is likely to involve relief from inhibition of activity of the first enzyme in the pathway. Lowering of the UDPGlcNAc concentration, perhaps "pulled" by the chitin synthetase reaction, is possible. In addition, other small molecular weight compounds, particularly when assayed in the presence of UDPGlcNAc, affect the activity of the first enzyme in zoospore extracts5. For example, uridine nucleotides, especially UTP and UDP, counteract the inhibitory effects of UDPGlcNAc (Fig. 6). Thus, an elevation of UTP concentration could have the same effect on relieving inhibition of the first enzyme as a depression in UDPGlcNAc concentration.

Post-translational regulation of biosynthetic pathways by end-product inhibition is, of course, a well documented general phenomenon. It has typically been interpreted in the context of physiological (metabolic) adaptation to changing cellular environments. The evidence discussed in this paper suggests that this type of regulation also operates as part of a normal developmental sequence. Hexosamine and chitin biosyntheses are "turned off" in the zoospore; their rapid "turn on" during germination accompanies the developmental transition from a phase where no cell wall is present to a phase where a chitin-containing wall is present. It is not inconceivable that in other developmental situations where very rapid changes in biosynthesis are called for, a similar type of regulation might be operative. It is worth underscoring that, for end-product inhibition of enzyme activity to be operative in a developmental sense, it would appear that utilization of the end product must also be subject to coordinated developmental control. Such appears to be the case in B. emersonii zoospores and during zoospore germination.