Developmentally regulated interconversions between end product-inhibitable and noninhibitable forms of a first pathway-specific enzyme activity can be mimicked in vitro by protein dephosphorylation–phosphorylation reactions

(enzyme regulation / feedback regulation / protein phosphorylation / Blastocladiella emersonii / hexosamine biosynthesis)

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ABSTRACT During the life cycle of Blastocladiella emersonii, dramatic shifts occur in the sensitivity of the first hexosamine biosynthetic pathway-specific enzyme [amidotransferase; 2-amino-2-deoxy-D-glucose-6-phosphate ketol-isomerase (amino-transferring), EC 5.3.1.19] to end product inhibition. These shifts are developmentally correlated with changes in the utilization of the end product (uridine-5'-diphospho-N-acetylglucosamine) for chitin synthesis (Selitrennikoff, C. P., Dalley, N. E. & Sonneborn, D. R. (1980) Proc. Natl. Acad. Sci. USA 77, 5998–6002). Alterations in amidotransferase sensitivity to end product inhibition can be mimicked by in vitro protein dephosphorylation–phosphorylation reactions, as follows: (i) Zoospore end product-inhibitable amidotransferase activity can be converted to a noninhibitable form by an endogenous (zoospore) protein phosphatase (phosphoprotein phosphophydrolase EC 3.1.3.16) reaction; this noninhibitable form can be converted back to an inhibitable form either by an endogenous CAMP-independent protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) reaction or with an added CAMP-dependent protein kinase. (ii) Noninhibitable amidotransferase activity from growing cells can also be converted to the inhibitable form with added protein kinase.

The zoospore of the water mold Blastocladiella emersonii is a highly compartmentalized, metabolically restricted cell. Two characteristic features of this cell are that it can move, by means of a posteriorly situated flagellum, and that it lacks a cell wall. The cell consumes energy, notably for movement and, probably, in resisting osmotic lysis (1), but zoospore biosynthetic activities, including RNA and protein synthesis, are very low to nonexistent (see ref. 2 for review). Early during zoospore germination, movement ceases and prior to the onset of other biosynthetic activities, a cell wall is abruptly constructed. Chitin, the major macromolecular component of the B. emersonii cell wall (3), is not detectable in the zoospore. However, accompanying the appearance of the initial cell wall, chitin suddenly accumulates to about 1.5–2% of the cellular dry weight; both the initial cell wall and chitin appear on schedule in the presence of concentrations of cycloheximide that effectively inhibit protein synthesis (4). Preexisting chitin synthase (UDP-2-acetamido-2-deoxy-D-glucosylchitin 4-β-acetamido-2-deoxy-D-glucosyltransferase, EC 2.4.1.16) is rapidly activated during this initial de novo wall construction, though the mechanism of activation remains in question (4–8). Particularly germane to this paper are the findings (4, 5, 9) that: (i) the zoospore contains too little substrate [uridine-5'-diphospho-N-acetylglucosamine (UDPGlcNAc)], by an order of magnitude, to account for the amount of chitin accumulated as the initial wall is formed; and (ii) this low level of UDPGlcNAc is nevertheless sufficient to inhibit its own biosynthesis, by means of feedback inhibition specifically of the first hexosamine biosynthetic pathway-specific enzyme—i.e., amidotransferase [2-amino-2-deoxy-D-glucose-6-phosphate ketol-isomerase (amino-transferring), EC 5.3.1.19].

Amidotransferase activity undergoes a dramatic decrease in sensitivity to UDPGlcNAc inhibition during zoospore germination and remains effectively insensitive throughout the growth phase, during which net chitin synthesis keeps pace with the exponential increase in mass. Sensitivity to end product inhibition returns during sporulation, a phase during which net chitin accumulation is not observed (5). Thus, there appears to be regulatory coupling during the life cycle between the extent of UDPGlcNAc biosynthesis and its consumption in chitin synthesis; when net chitin and cell wall accumulation occur (growth phase), UDPGlcNAc does not effectively inhibit its own synthesis and, conversely, when net chitin synthesis is curtailed (zoospore and sporulation phases), effective end product (UDPGlcNAc) inhibition of the hexosamine biosynthetic pathway is observed.

Because of the rapidity of the regulatory changes leading to de novo cell wall formation during zoospore germination, and because the changes occur on schedule in the presence of cycloheximide (4), we were led to explore posttranslational mechanisms effecting the conversion from end product-inhibitable (zoospore) to noninhibitable (germinating or growing cell) amidotransferase activity. We present evidence that this change can be effected in vitro by means of a protein phosphatase (phosphoprotein hydrolase, EC 3.1.3.16) reaction and that the reverse conversion, from noninhibitable to inhibitable enzyme activity, can be effected by a protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) reaction. The latter conversion can be effected by using either the zoospore enzyme preparation, after a phosphatase reaction, or the noninhibitable enzyme preparation from growing cells.

MATERIALS AND METHODS

Materials. Beef heart CAMP-dependent protein kinase and rabbit muscle protein kinase inhibitor were purchased from Sigma. Other materials were reagent grade from standard sources.

Growth Conditions and Enzyme Extraction. B. emersonii zoospores were harvested from cultures grown on peptone/
yeast extract/glucose agar plates and stored as lyophilized powders, as described (4). Growth-phase cells were prepared as described (10) after adding $8 \times 10^{10}$ zoospores to 14 liters of defined growth medium (11) containing 7 g of dextran sulfate. After 6 hr of growth at 27°C, the cells were collected on Whatman 541 paper and washed with 5 liters of distilled water. Cells were resuspended in 200 ml of distilled water and centrifuged at 220 $\times$ g for 9 min. Pellets were frozen in liquid nitrogen, lyophilized, and stored desiccated at $-20^\circ$C. Extracts were prepared by suspending zoospores or growth-phase cells in 50 mM 3-(N-morpholino)propanesulfonic acid (Mops) or 100 mM potassium phosphate buffer, pH 7.0, containing 10 mM glutamine, 1 mM KCl, and 600 mM sucrose. For each ml of incubation mixture desired, 8 mg of lyophilized zoospores was disrupted in 1 ml by repeated flushing through a Pasteur pipette. Approximately $2.5 \times 10^7$ growth-phase cells were used per ml of final assay mixture. The latter cells were disrupted in 1.5 ml with a Bronson Sonifier Cell Disruptor by using 15 10-sec bursts at 40 W with 30-sec cooling intervals. Extracts were centrifuged at 14,000 $\times$ g for 20 min and the supernatant fluids were subjected to Sephadex G-25 column (10.5 $\times$ 1 cm) chromatography as described (9) to remove endogenous low molecular weight compounds. Protein concentrations were determined by the Lowry method (12) with bovine serum albumin, fraction V, as standard.

**Incubations Prior to Amidotransferase Assays.** Preparations were incubated at 25°C and samples were withdrawn for amidotransferase assays at the times indicated in the figures. For protein phosphatase reactions, 5 mM MgSO$_4$ and 4 mM dithiothreitol were added to the incubation mixtures. For protein kinase reactions, the mixtures contained, in addition, 40 mM KF and 1 mM ATP; cAMP was 10 $\mu$M when present.

**Amidotransferase Assays.** At each incubation time point, samples were assayed for amidotransferase activity in the absence and presence of 500 $\mu$M UDPGlcNAc. The assays were performed as described (8) in 200-ml total volumes containing 1.2 $\mu$mol (6 mM) of fructose 6-phosphate and the same final concentrations of other chemicals as used in the extract preparations. Sample volumes added to reaction mixtures (30–50 $\mu$l) and reaction times (15–25 min) at 25°C were chosen to yield absorbances in the range of 0.2–0.7 in the absence of UDPGlcNAc, because percent inhibition of enzyme activity by 500 $\mu$M UDPGlcNAc was constant over this range. Zero time assay values were subtracted from the 15- to 25-min values. Examples of separate plots for amidotransferase activities assayed in the absence or presence of UDPGlcNAc are presented in Fig. 1. In the succeeding figures, data are presented in terms of percent enzyme activity inhibited by UDPGlcNAc, as described in the legend of Fig. 2A. To document reproducibility of the experiments in Figs. 2–4, data from repeat experiments are summarized in the figure legends with regard to specific amidotransferase activities, changes during the preassy incubations in uninhibited amidotransferase activities, and changes during the corresponding incubations in percent inhibitable amidotransferase activities. Values are given $\pm$SD.

**RESULTS**

In our previous studies, extracts were prepared and assayed for amidotransferase activity in solutions containing phosphate buffer, 500 $\mu$M UDPGlcNAc largely inhibited (ca. 90%) zoospore amidotransferase activity (refs. 4, 5, and 9; see also ref. 13) but only weakly inhibited growing cell amidotransferase activity (5). In the experiments to be considered, extracts were incubated for various times prior to amidotransferase assays. When incubated in the previously standard phosphate extrac-
cAMP-dependent protein kinases (15–17) was added during the second incubation period in experiments of the type just considered; the inhibitor did not affect the rate of return of inhibitable enzyme activity (Fig. 3B). To demonstrate that the concentrations of cAMP and of protein kinase inhibitor added were in effective ranges, a commercially available cAMP-dependent protein kinase was added to incubation mixtures. In the presence of 10 μM cAMP, amidotransferase inhibitory activity returned more quickly with the kinase added than with endogenous kinase activity alone; nevertheless, in the presence of the kinase inhibitor, the rate of return of amidotransferase inhibitory activity was reduced to that comparable to endogenous kinase activity (Fig. 3B). Thus, the concentrations of cAMP and protein kinase inhibitor used were effective in the incubation mixtures. For the following reasons, it is most unlikely that the cAMP-dependent protein kinase originated, during extraction from a dissociated catalytic subunit of a cAMP-dependent protein kinase: (i) the protein kinase inhibitor used can interact directly with such dissociated catalytic subunits in other systems (e.g., see refs. 16 and 17); and (ii) with histones used as substrates, a B. emersonii zoospore extract protein kinase activity that was indeed both kinase-inhibitor-sensitive and cAMP-independent has been reported (18).
In the above experiments, the zoospore end product-inhibitable amidotransferase was first converted to the noninhibitable form in vitro (protein phosphatase reaction) before conversion to the inhibitable form. We next sought to determine whether noninhibitable amidotransferase from an in vitro source (growing cells) could also be converted to an inhibitable form by an in vitro protein kinase reaction. Growth-phase extracts have not displayed endogenous protein kinase activity towards their endogenous, noninhibitable amidotransferase (Fig. 4). However, addition of the cAMP-dependent protein kinase to such extracts has resulted in extensive appearance of inhibitable amidotransferase activity. This change was not observed in the absence of added cAMP or in the presence of the cAMP-dependent protein kinase inhibitor. These results have thus furnished a critical piece of evidence; an in vitro protein kinase reaction can convert an in vitro source, as well as an in vitro produced form, of noninhibitable amidotransferase to the inhibitable form.

**DISCUSSION**

Previous data had documented that sharp regulatory changes in the hexosamine biosynthetic pathway occur naturally during the *B. emersonii* life cycle, in developmental coordination with chitin synthesis (5). A major site for these regulatory changes appears to be the first pathway-specific enzyme, the amidotransferase, which displays corresponding alterations in its sensitivity, or insensitivity, to pathway end product (UDPGlcNAc) feedback inhibition (5). The data presented here indicate that the alteration from sensitivity to insensitivity can be effected by enzyme-catalyzed protein dephosphorylation and that the alteration from insensitivity to sensitivity can be effected by enzyme-catalyzed protein phosphorylation. It must be emphasized that we have not yet distinguished whether the functional protein dephosphorylations–phosphorylations take place on the amidotransferase molecules themselves or on some other protein(s) (regulatory polypeptides or enzymes) that, in turn, determine the sensitivity or insensitivity of amidotransferase to end product inhibition. In either case, the question must be raised as to whether the in vitro catalyzed amidotransferase interconversions have physiological relevance. At the moment, the strongest evidence in favor of such a relationship is that either an in vitro desensitized form of amidotransferase (derived from the zoospore sensitive form) or a naturally insensitive form of amidotransferase (derived from growing cells) can each be converted to a sensitive form by an in vitro protein kinase reaction.

The question of physiological relevance can be considered further in the light of the evidence that zoospore extracts contain both protein phosphatase and cAMP-independent protein kinase activities that can serially effect the respective conversions of zoospore amidotransferase activity. The organism presumably favors the protein kinase reaction during sporulation, during which amidotransferase activity gradually converts from the end product-insensitive to the sensitive form. This conversion provides a means of preventing unrestricted UDPGlcNAc accumulation after its utilization in chitin synthesis is curtailed, during sporulation (see ref. 5). In zoospores, either the effective cAMP-independent protein kinase reaction predominates over the protein phosphatase reaction or both enzyme activities are rendered nonfunctional in vitro with respect to amidotransferase activity, because the latter enzyme remains in the inhibitable form. During zoospore germination, amidotransferase activity changes from the inhibitable to the noninhibitable form (5), suggesting that the protein phosphatase reaction then predominates over the protein kinase reaction.

Protein phosphatase activities have not previously been investigated in *B. emersonii*. Most of the previously published data concerning protein kinase activities in this organism (18, 19) cannot be easily related to the present data because proteins other than endogenous phosphate acceptors were added as substrates for protein kinase reactions. However, the comparatively low protein kinase activities observed in extracts from sporulating cells (19), zoospores (18), or growing cells (19) with only endogenous substrates were, in each case, predominantly cAMP-independent. The data of Juliani and Maia (18), who used zoospore extracts fractionated by DEAE-cellulose chromatography, are of particular relevance. Using histone H1 as a substrate, they observed two peaks of protein kinase activity, one cAMP-dependent and sensitive to a mammalian cAMP-dependent protein kinase inhibitor and the other cAMP-independent but still sensitive to the kinase inhibitor (i.e., a presumed catalytic subunit of a cAMP-dependent form of the enzyme). Using casein as substrate, the same workers observed a single major peak of protein kinase activity, clearly separated from the other two peaks and both cAMP-independent and insensitive to the protein kinase inhibitor. The latter two characteristics coincide with those displayed by the zoospore protein kinase activity effective in converting endogenous desensitized zoospore amidotransferase activity to a form sensitive to end product inhibition (Fig. 3). Thus, although zoospore extracts presumably do contain all three forms of protein kinase activity, only the kinase inhibitor-insensitive, cAMP-independent form appears to be effective in mediating the in vitro return of zoospore amidotransferase sensitivity to end product inhibition.

Two caveats remain concerning this conclusion: (i) a cAMP-dependent protein kinase from beef heart tissue, rather than from *B. emersonii*, can also mediate the above in vitro return (Fig. 3B); and (ii) as mentioned previously, conversion from noninhibitable to inhibitable amidotransferase activity occurs in vitro during the sporulation phase, as zoospores are being formed.

![Fig. 4. Sensitization of growing cell amidotransferase to end product inhibition.](image-url) Extracts from 6-hr growing cells were prepared in phosphate extraction buffer. Incubation mixtures contained, in addition, 5 mM MgSO4, 4 mM dithiothreitol, cAMP-dependent protein kinase at 1,000 units/ml and either no additions; △, 10 μM cAMP; or ▲, 10 μM cAMP plus protein kinase inhibitor at 1,000 units/ml. Enzyme assays were performed as in Fig. 1. Zero-time specific enzyme activities under the three incubation conditions were 35.5 ± 1.5 nmol of GlcN6P per min per mg of protein. Comparably lower specific enzyme activities with growing cell extracts than with zoospore extracts (previous figure legend) have been reported previously (5). The percent changes in uninhibited enzyme activities over the 60-min incubations were: ○, +8 ± 8 (two experiments); △, +6 ± 3 (three experiments); and ▲, −0.5 ± 14.5 (two experiments). The changes in percent enzyme activity inhibitable by 500 μM UDPGlcNAc were ○, −2 ± 10.6; △, +56.5 ± 10; and ▲, −1.5 ± 6.5.
thus, it remains possible that the relevant protein kinase during sporulation is not identical with the protein kinase activity characterized in extracts of fully formed zoospores. These caveats notwithstanding, the sporulation phase conversion from noninhibitable to inhibitable amidotransferase activity would appear to involve the predominance of protein kinase reaction(s) over protein phosphatase reaction(s); we are mindful of reports in other systems (e.g., ref. 20) of protein kinase-activated protein phosphatase inhibitors.

With regard to the conversion of inhibitable to noninhibitable amidotransferase during germination and the maintenance of the noninhibitable form throughout the growth phase, we have suggestive evidence that growing cells may contain inhibitor(s) of the relevant protein kinase activity; in mixed extract experiments, the otherwise effective zoospore protein kinase activity has failed to convert growing cell amidotransferase from the noninhibitable to the inhibitable form (unpublished data). Recall also from Fig. 4 that the growing cell extract failed to display protein kinase activity toward its own amidotransferase, but that a mammalian cAMP-dependent protein kinase did convert growing cell amidotransferase from the noninhibitable to inhibitable form. Most previously reported protein kinase inhibitors, including the one tested here, are effective against catalytic subunits of only cAMP-dependent protein kinase. However, heat-stable, cAMP-independent protein kinase inhibitors have also been recently reported (21, 22).

In this report, we have implicated protein phosphorylation–dephosphorylation reactions in the end product inhibitable–noninhibitable interconversions of amidotransferase activity during the B. emersonii life cycle. An impressive variety of other metabolic activities follow the same general life cycle pattern as the hexosamine biosynthetic pathway—i.e., severe depressions during sporulation, resulting in virtually complete inactivity in the zoospore, and abrupt activations during zoospore germination (see ref. 2 for review). Thus, it is certainly worth exploring the possibility that the functions of other key proteins are also regulated by similar life cycle phase-specific protein phosphorylation–dephosphorylation reactions. The work reported here demonstrates that it is feasible to use endogenous protein substrates, as well as endogenous (zoospore) protein kinase and protein phosphatase enzymes, to examine such post-translational regulatory changes.

This work builds on previous discoveries of the life cycle phase-dependent interconversions in amidotransferase sensitivity, or insensitivity, to end product inhibition, documented in our laboratory by Norman Dalley and Claude Selitrennikoff. We thank Tom Martin for sharing with us his expertise and knowledge concerning protein phosphorylation–dephosphorylation reactions. Diligent technical aid was contributed by Mike Strassman. We gratefully acknowledge the financial support of National Science Foundation Grant PCM 15334 (to D.R.S.) and of a postdoctoral traineeship from National Institutes of Health Grant H4000409 (to P.S.F.).