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Cyclic changes in translation of the mitochondrial isoenzyme account for the rhythm of aspartate aminotransferase activity in *Leptosphaeria michotii*

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Aspartate aminotransferase in *Leptosphaeria michotii* has previously been shown to have an activity rhythm in constant conditions. The enzyme is present as two isoforms whose levels were quantified along the activity rhythm by ELISA, using specific polyclonal antibodies raised against polypeptides purified to a state of apparent homogeneity. The time-course of the level of the cytosolic isoform remained unchanged along the experiment. On the contrary, the cyclic variations in amount and transaminase activity (using cysteine sulfinate as substrate) of the mitochondrial isoform gave rise to the aspartate aminotransferase activity rhythm of the fungus. The mRNA levels of the two isoforms, as determined by in vitro heterologous translation, remained monotonous along the daily cycle. These results and the sensitivity of the rhythm towards protein synthesis inhibitors are consistent with the hypothesis that the aspartate aminotransferase activity rhythm in this species is caused by some mechanism controlling the efficiency of translation of mitochondrial isoform mRNA.

Aspartate aminotransferase; Rhythm; Translation; (*Leptosphaeria michotii*)

1. INTRODUCTION

The sporulation rhythm expressed by the filamentous fungus *Leptosphaeria michotti* (West) Sacc. fed with asparagine as nitrogen source, has been shown to be regulated at three levels: (i) in nuclear DNA transcription [1]; (ii) in protein translation on 80S ribosomes [1,2]; (iii) in the asparagine-aspartate-pyruvate pathway, which controls the nitrogen flux and determines periodic variations of the intracellular pool of aspartate. Three enzymes of this pathway, namely asparaginase and aspartate and alanine aminotransferases express activity rhythms having a period identical with that of the sporulation rhythm [3]; the coordinated action of these enzymes is necessary for a normal expression of the sporulation rhythm [2,4]. Asparaginase, the first element of the asparagine-pyruvate pathway, has been shown to be regulated through reversible phosphorylation [5,6]. AAT, the second element of the pathway, is present as two isoforms, a cytosolic and a mitochondrial form, which were purified to a state of apparent homogeneity as holoenzymes alone or associated with specific proteins, and localized in situ using specific polyclonal antibodies [7]. Their regulation is under study. Our interest was first to determine if the cyclic transaminase activity was controlled at a transcriptional level or if it was due to a rhythm either in the quantity or in the specific activity of the enzyme.

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Abbreviations: AAT, aspartate aminotransferase (EC 2.6.1.1); BSA, bovine serum albumin; NaCl/P_i, phosphate-buffered saline

2. MATERIALS AND METHODS

2.1. *Reagents*

96-well polystyrene microtiter plates were from Linbro (Flow Lab., Puteau, France). Anti-rabbit Ig, β-galactosidase-linked species-specific F(ab')$_2$ fragment from donkey, Hybond-mAP (messenger affinity paper) and L-[4,5-^{3}H]-leucine (4.4 TBq/mmol) were from Amersham. Wheat germ translation system was from Promega and anisomycin from Sigma. All other chemicals were of analytical grade.

2.2. *Culture conditions*

L. michotti (West) Sacc. was grown in free-running conditions (at 23°C in the dark) as in [8]. For each time-course assay, cultures were harvested every 4 h, liquid-N_2-fixed, and lyophilized, or directly frozen and stored at −80°C for RNA isolation. Aspartate aminotransferase and cysteine-sulfinate transaminase activities and the polypeptide amounts of the cytosolic (A) and the mitochondrial (B) isoforms were then determined for each lot of cultures, and compared with translatable mRNA levels of isoforms A and B, isolated from frozen material. For studies of protein synthesis inhibitor effects, anisomycin (20 μM) was introduced into 5 day-old culture flasks, where it was present continuously, as in [4].

2.3. *Extraction and purification of aspartate aminotransferase isoforms, and antisera obtention*

Isoforms A and B were purified and rabbit specific antisera obtained as decribed in [7].

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2.4. *Quantitative estimation of aspartate aminotransferase isoenzymes*

The quantities of transaminases A and B present in *L. michotti* crude extracts or in the in vitro translation reaction mixtures were measured by ELISA at 405 nm, using anti-rabbit IgG, β-galactosidase-linked F(ab')$_2$ fragment from donkey and *O*-nitrophenyl-β-D-galactopyranoside as substrate.

2.5. *Western blot analyses*

Polypeptides fractionated by Laemmli polyacrylamide gel electrophoresis [7] were transferred to nitrocellulose sheets by electroblotting using the LKB Transblot unit, with a transfer time of 1 h at 0.8 mA/cm^2. Visualization of antibodies was performed, using the anti-rabbit Ig, β-galactosidase-linked F(ab')$_2$ fragment and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside as substrate, as described in [9].

2.6. *Isolation of poly(A$^+$) RNA*

We used the phenol-chloroform-NaDodSO$_4$ method of Lizardi [10], adapted to our material. Frozen hyphae were powdered with a pestle and mortar containing liquid nitrogen. Two vols of boiling 200 mM sodium borate/30 mM EDTA/1% NaDodSO$_4$ (pH 9.0) and the same volume of phenol saturated with 100 mM Tris (pH 8.0) were added. The phenol extraction was made four times; the last aqueous phase was mixed with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1,v/v/v). After centrifugation, the RNA solution was made up to 300 mM in sodium acetate (pH 7.0) and precipitated by ethanol. The RNA was washed with 70% ethanol and dissolved in water. To remove DNA and low M_r RNA, one vol. of 4 M LiCl was added and the solution placed on ice for 3 h. After centrifugation, the pellet was dissolved in water and the amount of RNA determined by its absorbance at 260 nm. The poly(A$^+$) RNA was partially purified by chromatography of RNA on the messenge affinity paper Hybond mAP from Amersham. Poly(A$^+$) RNA was stored precipitated in ethanol.

2.7. *In vitro translation*

In vitro translations were performed for 2 h at 25°C with micrococcal nuclease-treated wheat germ extract in 50-μl reaction mixtures containing 0.5–4 μg of poly(A$^+$) RNA and 925 kBq of L-[4,5-^{3}H]-leucine. The relative amount of total incorporated radioactivity was determined by counting alkali-resistant trichloroacetic-insoluble aliquots spotted onto Millipore glass fiber filters. Amounts of transaminase isoforms synthesized were estimated directly in the reaction mixtures by ELISA.

2.8. *Enzyme assays*

Aspartate aminotransferase activity was assayed as described in [11].

The cysteine-sulfinate transaminase activity of the mitochondrial isoenzyme of AAT was evaluated spectrophotometrically in crude extracts by reading the increase of NADH formed after a two-step reaction, using glutamate dehydrogenase (EC 1.4.1.3) as coupling enzyme [12, modified]. Samples were incubated for 7 min at 37°C in a mixture containing 7.5 mM 2-oxoglutarate, 15 mM cysteine sulfinate, 10 μM pyridoxal-phosphate, 100 mM Tris-HCl, pH 8.6, in a final volume of 400 μl. The reaction was stopped by addition of 40 μl 1 M HCl + 60 μl 10% v/v H_2O_2, and subsequent heating at 85°C for 3 min. After centrifugation, a 25 μl aliquot of the supernatant was added to the following mixture: 100 mM Tris-HCl, pH 8.6, 100 μM ADP, 400 μM NAD$^+$, 66 nkat/ml glutamate dehydrogenase in a final volume of 550 μl, and incubatd at 37°C for 30 min. Absorbance changes were measured at 340 nm in a Kontron Uvikon SP 810 spectrophotometer.

2.9. *Protein measurement*

Protein was measured as described by Lowry et al. [13], or, in the case of a low protein concentration, by the method of Bradford [14]. BSA was used as standard.

3. RESULTS AND DISCUSSION

3.1. *Characteristics of the polyclonal antisera*

Rabbit antisera raised against AAT isoforms A and B were shown to be specific by reacting as single bands on Western blots of total proteins present in crude extracts separated under denaturing conditions by polyacrylamide gel electrophoresis (not shown). Antiserum raised against form A holoenzyme was not reactive against form B holoenzyme, and vice versa: the ELISA signal obtained with the heterologous reaction did not exceed 5% of the signal obtained with the homologous reaction. Using purified AAT holoenzymes A and B, the optimum signal for isoform A was obtained with the serum anti-AAT A diluted 1000 times and for isoform B with the serum anti-AAT B diluted 250 times. The standard curves resulted in a signal which was a linear function of the log of the amount of AAT from 5 to 65 ng for isoform A, and from 16 to 70 ng isoform B (fig.1a). Using crude extracts of *L. michotti*, the ELISA signa was a linear function of the log of the extract volume until 25 μl (fig.1b).

The polyclonal antisera used, therefore, appear to be useful in the quantitative determination of the AAT isoforms of *L. michotti* present in crude extracts or in in vitro translation analyses.

3.2. *Time-course of the levels of AAT isoforms A and B and their activities*

For each time-course assay, crude extracts were prepared from samples collected every 4 h, aspartate and cysteine-sulfinate transaminase activities measured, and quantities of the two AAT isoforms determined by ELISA.

These results are shown in fig. 2: (i) The aspartate AT activity (i.e the sum of activities of isoenzymes A and B) expressed a 20-h periodicity; (ii) The two-step reaction used permitted the measurement of the transaminase activity of isoenzyme B alone, using cysteine sulfinate as a substrate (this compound is not degraded

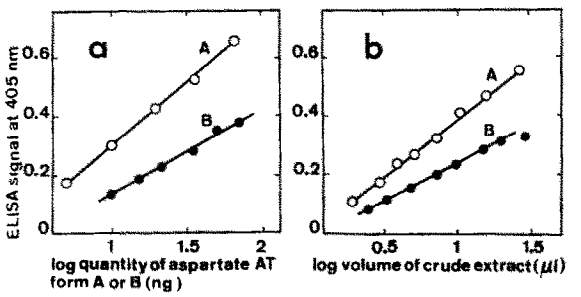

Fig.1. Standard calibration curves for aspartate aminotransferase isoforms A (○—○) or B (●—●). (a) ELISA signal at 405 nm is plotted vs log quantity of aspartate AT A (5–65 ng) or B (10–70 ng). Dilution of rabbit serum against AAT A: 1/1000, against AAT B: 1/250. (b) Relationships between ELISA signal at 405 nm and log volume (μ) of crude *L. michotii* extracts with anti-AAT A serum diluted 1/1000, or anti-AAT B serum diluted 1/250.

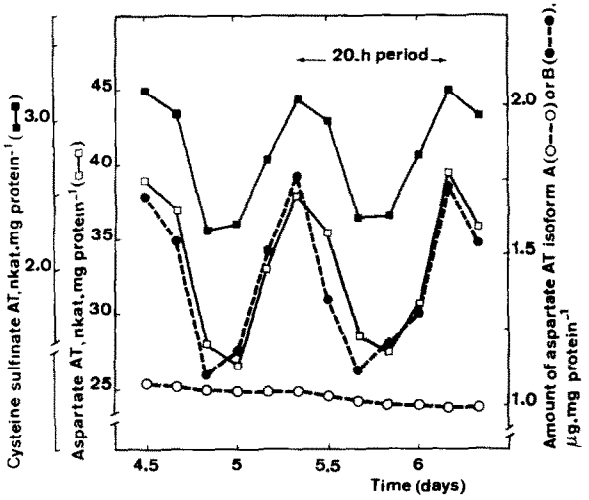

Fig.2. Time-course of total AAT activity (□—□) and cysteine sulfinate transaminase AT activity of isoform B alone (■—■), compared with the time-course of amounts of isoform A (○--○) and B (●--●) estimated by ELISA in crude *L. michotii* extracts. Experiments were repeated 4 times with similar results.

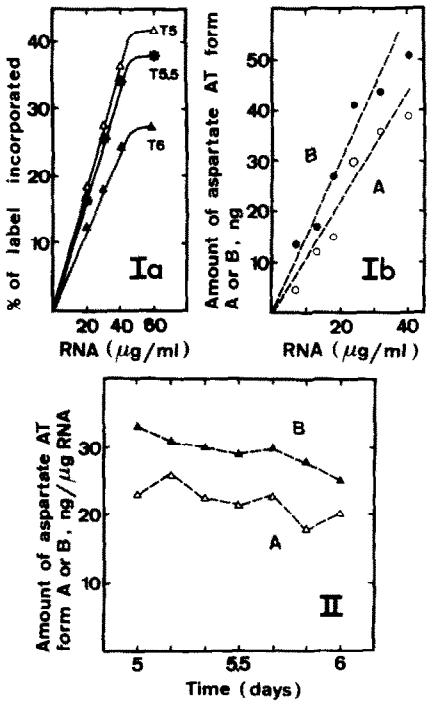

Fig.3. Time-course of amounts of translatable AAT isoform mRNAs, determined by translation in a wheat germ extract. (I) Effects of poly(A^+) RNA amount on: (a) [3,5-^{3}H]-leucine incorporation in total proteins. For 20 µg/ml of poly(A^+) RNA, the incorporation efficiency ranged from 12.5% for 6-day-old cultures (T6) to 18.5% for 5-day-old cultures (T5); (b) synthesized isoform A ((○--○) and B (●--●), determined by ELISA (µg/ml). (II) Time-course of isoform A (Δ--Δ) and B (▲--▲) amounts synthesized (ng/µg poly(A^+) RNA).

by isoform A, as shown earlier [7]). The cysteine sulfinate and aspartate transaminase activities oscillated with the same 20-h periodicity; (iii) The time-course of the level of isoform A remained monotonous and practically unchanged along the experiment; (iv) The time-course of the amount of isoform B was rhythmic and synchronous with the two rhythms of transaminase activity; the oscillation shapes were similar for the three rhythms. Moreover, the rhythm amplitudes (the percentage difference between minimum values and mean values) for the level of isoform B (22%) and cysteine sulfinate activity (17.3%) were very similar to that of aspartate AT activity (18.6%).

These results allow us to conclude that: (i) The cyclic variation of the aspartate AT activity in *L. michotti* is the result of the variation in the activity of the isoform B only; (ii) The activity rhythm is entirely accounted for by the cyclic variation in the amount of isoform B.

3.3. *Is the rhythm of the level of isoform B controlled at the transcriptional or translational level?*

The purified poly(A^+) RNAs were tested for AAT mRNA activity with the wheat germ system of translation, along the activity rhythm, by their capacity to synthesize isoform A and B polypeptides.

The incorporation of amino acids into total proteins was first examined as a function of the amount of added poly(A^+) RNA (fig.3Ia). The amount of newly synthesized proteins was a linear function of the poly(A^+) RNA up to a concentration of 50 µg/ml, after which protein synthesis was gradually inhibited. The amounts of newly synthesized AAT isoforms A and B were then determined by ELISA directly in the reaction mixtures, taking into account the differences in radioactivity incorporation resulting from the time of extraction and purification of the different poly(A^+) RNA samples (fig.3IB). These amounts were a linear function of added poly(A^+) RNAs up to a concentration of 40 µg/ml.

Accordingly, 20 and 30 µg/ml concentrations of poly(A^+) RNAs were used to follow the amounts of translatable AAT mRNAs along the transaminase activity rhythm, by ELISA of synthesized AAT A and B polypeptides (fig.3II). No great differences were observed in the amounts of translated AAT isoforms A and B. So, independently of the time of extraction, the AAT mRNAs are present in the same amount and translate equally well if a heterologous translation system is used. This excludes transcriptional control of mRNA as a possible origin of cyclic variation of the amount of isoform B in vivo. To explain this periodic variation, we still cannot define the respective roles of a periodic translation and of a rhythmic mobilization from an inactive state of the mRNA to its translatable form [15,16]. A periodic involvement of proteolytic events cannot be excluded. Nevertheless, no participation of an active protease has been observed during the purification of the two isoenzymes extracted at different phases of the activity rhythm [7].

3.4. *Effects of protein synthesis inhibitors on the amount and activity rhythms of the AAT isoforms.*

Our previous assays have pointed to the fact that protein synthesis on 80S ribosomes could be involved in the control of the AAT activity rhythm in *L. michotti*. In the presence of a cycloheximide concentration which completely stopped protein synthesis in the fungus, the AAT activity fell dramatically and the rhythm was suppressed [4]. Besides, cycloheximide concentrations which partially inhibited protein synthesis, modified an essential parameter of phototaxis rhythm, the period which was lengthened, in *Euglena gracilis* [17]. Moreover, high levels of anisomycin had the same effects as cycloheximide on protein synthesis and transaminase activity rhythm of *L. michotti*.

Expecting specific effects of the drug on the main parameters of periodicity, a level of anisomycin (20 μM), which did not affect ponderal growth and slightly depressed protein synthesis, was used. The drug essentially provoked an increase of period and of the cysteine sulfinate transaminase activity rhythm of isoform B, and a similar modification of the rhythm of the changes in the quantity of B. The level of isoform A was slightly depressed, but had a monotonous time-course (not shown).

These facts underline the outstanding role of protein synthesis regulation in the expression of AAT rhythmicity.

Periodicities in the expression of enzymes or proteins can be controlled by a circadian clock at different levels: transcription [18–20], translation [21–24] or post-translation [25,26]. *L. michotti* is the only simplified model known to date, in which two different regulatory processes control rhythmic changes of enzyme activity in the same metabolic pathway: post-translation (through reversible phosphorylation) for asparaginase [5,6] and translation for the mitochondrial isoform of AAT (the present results).

In short, we show in this paper the differing behavior of two AAT isoforms in *L. michotti*: the level of isoform B showing a cyclic variation and directly contributing to the activity rhythm, and isoform A, whose part in the periodic system is still to be defined. Obtention of cDNAs specific for the AAT isoforms of *L. michotti* is now in progress. These probes will permit direct estimation of the levels of mRNAs coding for the two isoforms over time by RNA (Northern) hybridizations, and the identification of factors modulating isoform-B mRNA translation.

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