Cyclic activity of L-asparaginase through reversible phosphorylation in *Leptosphaeria michotii*

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Asparaginase in *L. michotii* has previously been shown to have an activity rhythm, the mechanisms of which were investigated. In vitro activation, or reactivation after dephosphorylation, of the partially (200-fold) purified asparaginase with protein kinase activity was obtained by ATP or P_i addition; these effects varied according to the phase of the activity rhythm at which enzyme was extracted. A high-M_r aggregate with asparaginase activity was phosphorylated by [γ-^{32}P]ATP. By SDS-electrophoresis of dephosphorylated asparaginase a ~60-kDa ^{32}P-labelled protein with alkaline phosphatase activity became detectable. Regulation of the asparaginase activity rhythm in *L. michotii* is dependent on a reversible phosphorylation process.

Asparaginase *Protein kinase* *Alkaline phosphatase* *Rhythm* Leptosphaeria michotii

1. INTRODUCTION

The biochemical background of circadian rhythmicity is still little understood. To approach the mechanisms of such low-frequency oscillating systems, we analyzed the sporulation rhythm in *Leptosphaeria michotii* (West) Sacc. This rhythm has been shown to be regulated at two levels: (a) protein synthesis on 80 S ribosomes; (b) the asparagine-pyruvate pathway, 3 enzymes of which, namely asparaginase and aspartate and alanine aminotransferases, expressed activity rhythms having a period identical with that of the sporulation rhythm [1,2]. Asparaginase activity rhythm, which played a very important part in this periodic system, continued in crude extracts in spite of a dramatic inhibition of protein synthesis [3]; this rhythm was not suppressed in partially (200-fold) purified extracts mainly constituted of form I and form II asparaginases (130–140 kDa) and their polymers (270 and 500–600 kDa) and a ~60-kDa protein [4]. All these facts supposed that post-transductional protein modifications could be implicated in the regulation of the rhythmic asparaginase activity in *L. michotii*. We report here that the isolated protein complex is implicated in rhythmic asparaginase activity through a reversible phosphorylation process.

2. MATERIALS AND METHODS

2.1. *Materials*

L. michotii (West) Sacc. was grown as in [5] for 5.5 days by which time asparaginase activity rhythm was fully expressed. Fifty cultures were then harvested every 4 h and lyophilized. A partially (200-fold) purified asparaginase (L-asparagine amidohydrolase, EC 3.5.1.1) was obtained in each case as detailed in [4].

2.2. *Asparaginase assay*

Asparaginase was assayed at 25°C for 30 min and reaction products analyzed as in [2]. The standard assay medium (final vol. 1.1 ml) contained 100 mM Tris (Cl^-, pH 8.6), 10 mM [U-^{14}C]asparagine (spec. act. 0.1 μCi/μmol) and enzyme solution (20 μg protein).

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2.3. *Autophosphorylation of protein complex*

Protein kinase activity was assayed at 4°C for 10 min in a final volume of 1 ml containing 100 mM Tris (Cl^-, pH 8.6), 5 mM β-mercaptoethanol, 5 mM MgO, 10 μM [γ-^{32}P]ATP or $^{32}P_i$ (spec. act. 0.25 μCi/nmol) and enzyme solution (20 μg protein). Native and denatured forms of ^{32}P-labelled proteins were analyzed by electrophoresis on gradient polyacrylamide slab gels [4,6] after precipitation by 0.15 M trichloroacetic acid in the presence of 5 mM EDTA and bovine serum albumin (25 μg/ml) and centrifugation at 20000 × g for 30 min. They were detected by autoradiography at −80°C and their radioactivity determined by Cerenkov counting of the 30% perhydrol solubilized gels.

2.4. *Dephosphorylation of protein complex*

Protein complex (20 μg) was dephosphorylated at 4°C for 10 min in a total volume of 1 ml containing 100 mM Tris (Cl^-, pH 8.6), 10 mM $MgCl_2$, 10 mM $ZnCl_2$ and 1 μg activated calf-intestinal alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1).

2.5. *Direct detection of enzyme activity*

Direct detection of enzyme activities on polyacrylamide gels was performed for asparaginase by the formation of an insoluble complex of Na tetraphenylboron with NH_4^+ as in [7] and for alkaline phosphatase by the formation of calcium phosphate [8] detected as a nitrophosphomolybdo complex stained with methyl green [9].

3. RESULTS

3.1. *In vitro manipulation of asparaginase activity by phosphorylation and dephosphorylation*

The activity of form I asparaginase was increased by ATP, supplied in the presence of Mg^{2+}, irrespective of the phase of the in vivo activity rhythm at which enzyme extraction was performed, but the rhythm of asparaginase activity persisted. P_i addition completely replaced ATP-Mg^{2+} effects. The addition of ATP-Mg^{2+} or P_i to form II asparaginase resulted in similar effects (fig.1). Asparaginase activation by P_i was obtained without Mg^{2+} supply. So, the possible participation of a small quantity of protein-bound divalent cations in this reaction was verified using EGTA.

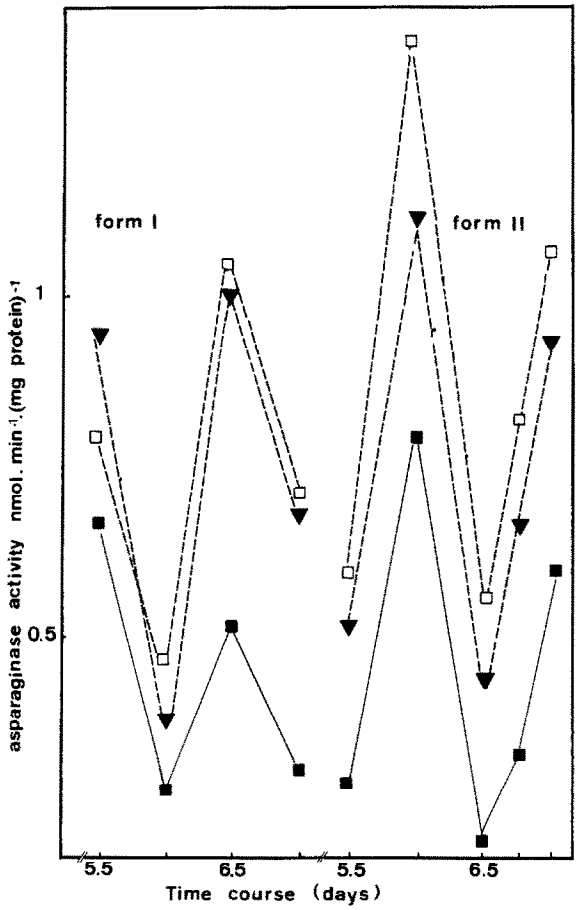

Fig.1. Time course of ATP-Mg^{2+} or P_i-dependent in vitro activation of *Leptosphaeria michotii* asparaginase form I [non-retained on DEAE–Sephacel column eluted with 50 mM Tris (Cl^-, pH 8.6)] and form II (eluted from the same column by an NaCl gradient). The enzyme, extracted from cultures aged from 5.5 to 7 days, was first incubated at 4°C for 10 min in 50 mM Tris buffer (pH 8.6) alone (■—■) or supplied with 10 μM ATP + 5 mM MgO (□---□), or with 66 mM P_i (▼---▼). Asparagine (10 mM) was then added and incubation performed at 25°C for 30 min for asparaginase assay. Experiments were performed 4 times with similar results.

Asparaginase activity was inhibited by 66% when EGTA was added not only to a Tris solution, but also to a phosphate solution able to activate by itself the enzyme. These EGTA inhibiting effects were completely reversed by Mg^{2+} (table 1).

The effects of a dephosphorylating process on asparaginase activity were analyzed by two means: calf-intestinal alkaline phosphatase; freezing.

Table 1

EGTA and Mg^{2+} effects on *Leptosphaeria michotii* asparaginase activity

Additions	Tris buffer	Phosphate buffer
None	100	233
Mg^{2+}	125	237
EGTA	33	33
Mg^{2+} + EGTA	117	115

Asparaginase was preincubated at 4°C for 10 min in 100 mM Tris (Cl^-, pH 8.6) or 66 mM phosphate (pH 8.6) supplied with 5 mM EGTA or 5 mM Mg^{2+}. Asparagine (10 mM) was then added and incubation performed at 25°C for 30 min for asparaginase assay. The results are expressed as a percentage of the original activity. Tests were performed in 6 replicates, single samples not differing more than 10% from each other

Alkaline phosphatase deactivation of a very active enzyme (issued from 5.5-day-old cultures) was very strong whereas it was weak in the case of a less active asparaginase (from 5-day + 16-h-old cultures). P_i reversed the alkaline phosphatase effect in such a way that asparaginase rhythmicity was almost completely suppressed (fig.2). Freezing always caused a significant loss of asparaginase activity, which was nullified by ATP-Mg^{2+} of P_i addition in thawed enzyme solutions, but the rhythm of asparaginase activity persisted (fig.3).

All these results, based on functional changes, indicate that the asparaginase used could be deactivated by an extrinsic and an intrinsic alkaline phosphatase and activated or reactivated by an intrinsic protein kinase.

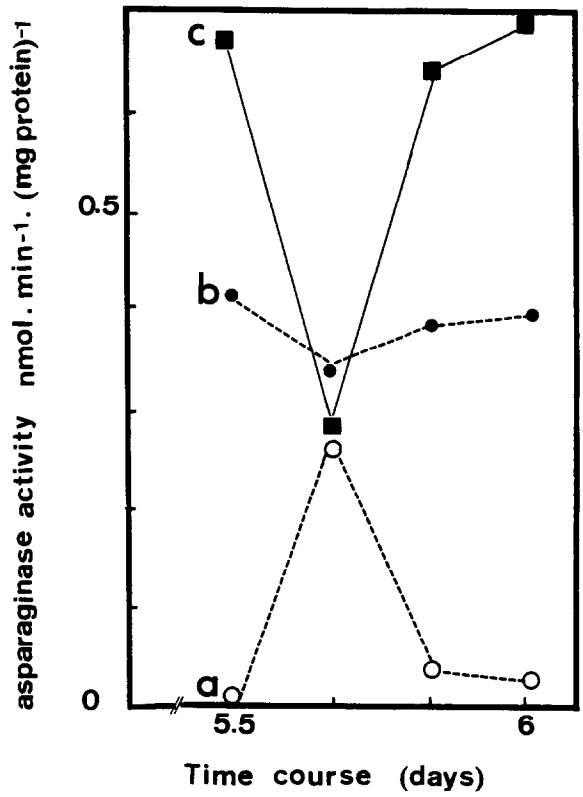

Fig.2. Time course of *Leptosphaeria michotii* asparaginase inactivation by calf-intestinal alkaline phosphatase and its reactivation by P_i. Purified asparaginase was preincubated at 4°C for 10 min with alkaline phosphatase (a); 66 mM P_i was introduced in the reaction mixture at the end of the preincubation period (b); control, non-dephosphorylated (c). Asparagine (10 mM) was then added and incubation done at 25°C for 30 min for asparaginase assay. Experiments were performed 4 times with similar results.

3.2. *In vitro protein phosphorylation*

Purified preparations of asparaginase were incubated with [γ-^{32}P]ATP and analyzed by electrophoresis. Under non-denaturing conditions, a portion of the radioactivity was found to be tightly associated with the high-M_r aggregate (2000–8000 cpm by spot, according to the experiments); asparaginase holomer and its dimer form were slightly labelled (200–300 cpm by spot) whereas the ~60-kDa protein remained unlabelled. Under denaturing conditions, holomer and dimer forms of asparaginase disappeared and a 34–36-kDa protein (the asparaginase subunit) was detected. Only the high-M_r protein aggregate was labelled (fig.4a). Incubation of asparaginase preparations with $^{32}P_i$ instead of [γ-^{32}P]ATP gave the same results (not shown). These data constitute preliminary evidence that an intrinsic protein kinase phosphorylates asparaginase molecules essentially.

3.3. *Protein dephosphorylation and rephosphorylation*

^{32}P-labelled protein complex was incubated with alkaline phosphatase and preparations analyzed by electrophoresis. Under non-denaturing conditions radioactivity, detected in the sole protein ag-

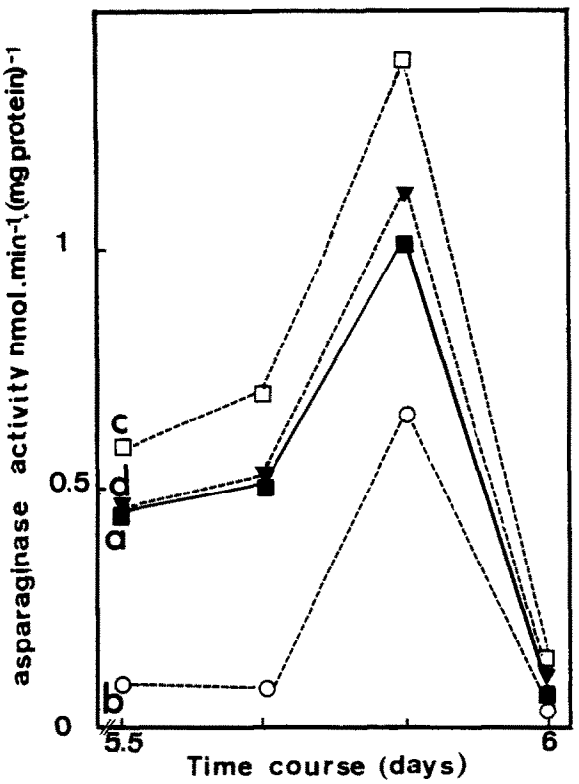

Fig.3. Freezing and phosphate effects on the time course of asparaginase activity: 50 mM Tris (Cl^-, pH 8.6) enzyme solutions were frozen at −20°C for 1 week then thawed and incubated at 4°C for 1 h before asparaginase assay. Control before (a) and after freezing (b). Thawed enzyme solutions preincubated in the presence of ATP-Mg^{2+} (c) or 66 mM P_i (d). Experiments were performed 3 times with similar results.

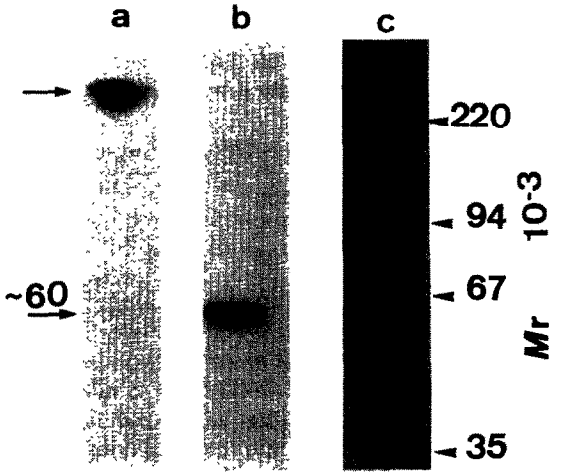

Fig.4. Autoradiographs of SDS linear 10–16% (w/v) acrylamide gel separation of partially purified asparaginase. Lanes: (a) phosphorylated by [γ-^{32}P]ATP-Mg^{2+}, (b) [^{32}P]phosphorylated and then dephosphorylated by calf-intestinal alkaline phosphatase, (c) dephosphorylated and then [^{32}P]phosphorylated. The general conditions of these assays are described in sections 2.3 and 2.4. M_r values have been determined using markers in parallel.

gregate, was increased. Under denaturing conditions ^{32}P was released from the protein aggregate and radiolabelling of the ~60-kDa protein was observed; alkaline phosphatase, which is constituted by two 70-kDa subunits [10], was not labelled in the present assays (fig.4b). When unlabelled asparaginase was first dephosphorylated by alkaline phosphatase and P_i added to stop the phosphatase activity before [γ-^{32}P]ATP addition, both protein aggregate and ~60-kDa protein were labelled (fig.4c). In fact, under dephosphorylating as well as rephosphorylating conditions, the relative radioactivity of the two spots was dependent on the phase of the asparaginase activity rhythm in which enzyme extraction was performed. Therefore, to obtain the ~60-kDa protein phosphorylation at the expense of the high-M_r aggregate it was necessary to add calf-intestinal alkaline phosphatase, which could act in this case as a protein transphosphorylase.

3.3. *Detection of enzyme activities in the* L. michotii *protein complex*

The presence of an intrinsic phosphatase is indirectly indicated by the data given in section 3.1. The phosphatase activity of native proteins isolated by electrophoresis was investigated in situ. The ~60-kDa protein was able to degrade β-glycerophosphate at pH 8.6 in the presence of 10 mM Mg^{2+}, thus showing alkaline phosphatase activity; it had no asparaginase activity (not shown).

4. DISCUSSION

Our results demonstrate that the regulation of asparaginase activity in *L. michotii* is dependent on a reversible phosphorylation process, the enzyme activity being increased under phosphorylating conditions and vice versa. To our knowledge

this is the first report dealing with this type of post-translational modification of a rhythm of enzyme activity. The partially purified protein complex, which was isolated from *L. michotii*, possesses protein kinase and alkaline phosphatase activities. Three proteins of this complex undergo in vitro phosphorylation: asparaginase, a ~60-kDa alkaline phosphatase and a high-M_r aggregate. The protein kinase involved is active in the presence of ATP or P_i and in both cases Mg^{2+} seemed to be necessary. Further characterization and purification of all constituents of the *L. michotii* protein complex, which are involved in the reversible phosphorylation process, are now under active investigation.

Calf-intestinal alkaline phosphatase is a non-specific phosphohydrolase, able to act as a phosphotransferase in the presence of the nucleophile acceptors, Tris, ethanolamine or glycerol [11]. Our results show that it might also act as a protein transphosphorylase, the phosphate acceptor being the *L. michotii* alkaline phosphatase. Asparaginase reactivation under phosphorylating conditions differed according to the deactivation process used. On treatment with calf-intestinal alkaline phosphatase the asparaginase activity rhythm disappeared whereas it persisted after freeze-thawing; in the latter case deactivation was supposed to be due to the sole *L. michotii* alkaline phosphatase effect. These facts underline the possible regulatory role of this molecule, similar to that of several protein phosphatases [12,13] but, in this case, in the control of an enzyme activity rhythm.

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