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Ammonium-sensitive protein kinase activity in plasma membranes of the cyanobacterium *Anacystis nidulans*

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Abstract

Cytoplasmic membranes prepared from nitrate-grown *Anacystis nidulans* cells exhibit a Mg^{2+}-dependent protein kinase activity able to phosphorylate in vitro plasma membrane polypeptides with molecular masses of 98, 93, 83, 47, 44 and 31 kDa. The protein kinase activity was inhibited in cytoplasmic membrane preparations from nitrate-grown cells which had been exposed to ammonium for 5 min. Parallely, ammonium exposure also resulted in a more than two-fold activation of an alkaline phosphatase activity present in the soluble fraction. These results are discussed in relation to the well-known inhibition by ammonium of nitrate transport activity, and a hypothesis for the regulatory mechanism involved is presented.

Key words: Protein phosphorylation; Plasma membrane protein kinase; Ammonium signal; Nitrate transport; *Anacystis, Synechococcus*

1. Introduction

Post-translational modification of proteins by phosphorylation/dephosphorylation plays an essential role in the control of metabolism in prokaryotes, as it does in eukaryotes. The protein kinases and protein phosphatases that catalyse the reversible phosphorylation of target systems, such as enzymes or transmembrane nutrient transport, allow immediate and effective responses of cells to internal and external environmental changes, prior to transcriptional responses mediating longer-term adaptations to such environmental alterations [1–3]. In cyanobacteria, protein kinase activities were first detected in cell-free extracts of the N_2-fixing strain *Anabaena* PCC 7120. At least 12 polypeptides in the soluble fraction, and 3 polypeptides of 46, 43 and 29 kDa in the particulate fraction were phosphorylated in vitro at the expense of [γ-^{32}P]ATP, the pattern of phosphorylation being sensitive to intermediary metabolites such as glucose 6-phosphate [4]. Glucose and light also modulate in vivo phosphorylation of soluble and membrane proteins in parallel to modulation of inorganic carbon transport in the unicellular non-N_2-fixing strain *Synechocystis* [5]. In *Synechococcus* 6301, reversible light-dependent protein phosphorylation was observed in isolated thylakoid membranes, affecting mainly two polypeptides of 18.5 and 15 kDa [6]. Light also effected the phosphorylation of soluble proteins of 19 and 13 kDa [7]. This 13-kDa polypeptide, which has been identified with the *glnB* gene product (P_{II} protein), was shown to be phosphorylated in vivo at Ser residues. P_{II} was highly labelled in the presence of nitrate or of light favoring PSII excitation, while ammonium or light favoring PSI excitation decreased the phosphorylation status of P_{II}. This has led to the proposal that cyanobacterial P_{II} might act, as in enteric bacteria, as a central signal transmitter of the N-status of the cell [7–8].

Nitrate is the major source of nitrogen for non-N_2-fixing cyanobacteria. The rate-limiting step of nitrate assimilation is nitrate transport into the cell, an active process driven by the energy of the $\Delta\mu Na^+$ across the plasma membrane [9–11]. In *Anacystis (synechococcus)*, ammonium prevents the cotranscription of a cluster of genes (*nirA-narB*) encoding for proteins of the nitrate reducing system as well as for proteins involved in nitrate transport [12–14]. In addition to this control at the transcriptional level, ammonium elicits an effective and reversible inhibition of nitrate transport activity. When nitrate-grown cells actively transporting nitrate are exposed to ammonium, nitrate transport activity is completely inhibited whithin seconds [9,15]. The mechanism by which ammonium effects this rapid and reversible inhibition of nitrate transport activity, as well as the nature of the regulatory signals involved, are still unknown [16,17]. Here, we present results showing that the cytoplasmic membranes of nitrate-grown *A. nidulans* cells contain a Mg^{2+}-dependent protein kinase activity able to phosphorylate in vitro several plasma membrane polypeptides including a 47-kDa protein previously shown to be involved in nitrate transport [18–19]. The

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Abbreviations: HEPES, *N*-(hydroxyethyl)piperazine-*N'*-2-ethan esulfonic acid; *p*NPP, *p*-nitrophenylphosphate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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plasma membrane protein kinase activity became inhibited, while a soluble alkaline phosphatase activity became activated, after exposure of the cells to ammonium. The results suggest that nitrate transport activity might be controlled by a mechanism of protein phosphorylation/dephosphorylation

2. Materials and methods

2.1. Culture conditions and cell fractionation

Anacystis nidulans (*Synechococcus leopoliensis* 1402–1 from Göttingen University, Germany) was grown photoautotrophically with nitrate as nitrogen source to a cell density of 15–20 μg chlorophyll *a*/ml as described previously [18]. Cells were harvested by centrifugation at room temperature (8000 × *g*, 10 min). Cytoplasmic membranes and soluble fraction of the cells were prepared, with the indicated minor modifications, according to the procedure by Omata and Ogawa [20], which includes lysozyme treatment with 0.5 mM EDTA, French press disruption of cells, and ultracentrifugation (130,000 × *g*, 18 h, 4°C) in a sucrose step gradient. The cytoplasmic membrane fraction (orange band) was focused at 30% sucrose, the thylakoid membrane fraction (dark green band) at the interface 39–50% sucrose, and the soluble fraction, containing fluorescent purple phycobiliproteins remained in the 50% sucrose layer.

The cytoplasmic membrane fraction was collected, washed in three volumes of 10 mM TES-NaOH buffer, pH 7.0, containing 10 mM NaCl, and sedimented by centrifugation (150,000 × *g*, 2 h, 4°C). The pellet was resuspended in a small volume of the same buffer. After determining protein according to Bradford [21], 80 mM each DTT and Na_2CO_3were added to the membrane preparation, which was divided into aliquots and immediately frozen and stored.

The soluble fraction was also collected, diluted in three volumes of the above buffer and centrifuged (150,000 × *g*, 2 h, 4°C) to eliminate contaminating thylakoid membranes. The blue supernatant was collected and, after protein determination, aliquots were frozen.

Exposure of cells to ammonium was performed under culture conditions, by adding 5 mM NH_4Cl to the nitrate-growing cells 5 min before starting the cell fractionation procedure, maintaining ammonium present during lysozyme treatment and cell disruption.

2.2. Protein kinase assay

Protein kinase activity was assayed by incubating at 30°C an amount of cytoplasmic membranes equivalent to 60 μg of protein in a reaction mixture containing, in a volume of 0.03 ml, 23 mM TES-NaOH buffer, pH 7.0; 5 mM $MgCl_2$ and 0.11 μM [γ-^{32}P]ATP (110 TBq/mmol, Amersham). The reaction was started by addition of [γ-^{32}P]ATP, and after 30 min stopped by adding 10 μl of electrophoresis sample buffer (125 mM Tris-HCl, pH 6.8; 4% (w/v) SDS; 20% (v/v) glycerol; 10% (v/v) 2-mercaptoethanol and 0.01% (w/v) Bromophenol blue), and boiling for 5 min. When required, [γ-^{32}P]ATP was replaced by [α-^{32}P]ATP, and $MgCl_2$ was supressed or replaced by $CaCl_2$.

The denatured samples were subjected to SDS-PAGE according to the procedure of Laemmli [22] in 7.5–15% polyacrilamide-SDS slabs gels. Molecular mass standards from BioRad (phosphorylase *b*, 92.5 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa, and lysozyme, 14.4 kDa) were run in each assay. Protein detection was performed by Coomassie blue R-250 staining. Radioactive polypeptides were detected by autoradiography on Hyperfilm (Amersham) exposed for 2 days at −80°C.

2.3. Alkaline phosphatase assay

Alkaline phosphatase activity was assayed at 30°C with *p*-nitrophenylphosphate (*p*NPP) as substrate [23], in a reaction mixture (1 ml) containing 25 mM Tris-HCl buffer, pH 8.0; 20 mM KCl; 30 mM $CaCl_2$; soluble fraction equivalent to 60 μg of protein, and variable concentrations (from 1 to 50 mM) of *p*NPP. Production of *p*-nitrophenol was determined by monitoring absorbance at 420 nm.

3. Results

3.1. Protein kinase activity in the plasma membrane of A. nidulans

When cytoplasmic membranes prepared from nitrate-grown *A. nidulans* cells were incubated with [γ-^{32}P]ATP and $MgCl_2$ prior to polypeptide analysis by SDS-PAGE, several radioactively labelled bands were detected by autoradiography. Fig. 1 (lane B) shows the six major labelled polypeptides, with molecular masses of 98, 93, 83, 47, 44, and 31 kDa, which were consistently observed in most preparations. Three of these bands, those of 98, 93 and 83 kDa, were hardly visible after Coomassie blue staining, although they were heavily labelled. The 47, 44, and 31 kDa labelled bands were, however, readily stained by Coomassie blue (Fig. 1, lane A). Interestingly, the 47-kDa labelled polypeptide comigrated with that previously identified as an essential component of the nitrate transport system in *A. nidulans* [18–19]. In order to assess whether the labelling of these cytoplasmic membrane polypeptides corresponded to protein phosphorylation or to ATP-binding, the membranes were incubated with [^{32}P]ATP labelled in either the α or the γ position.

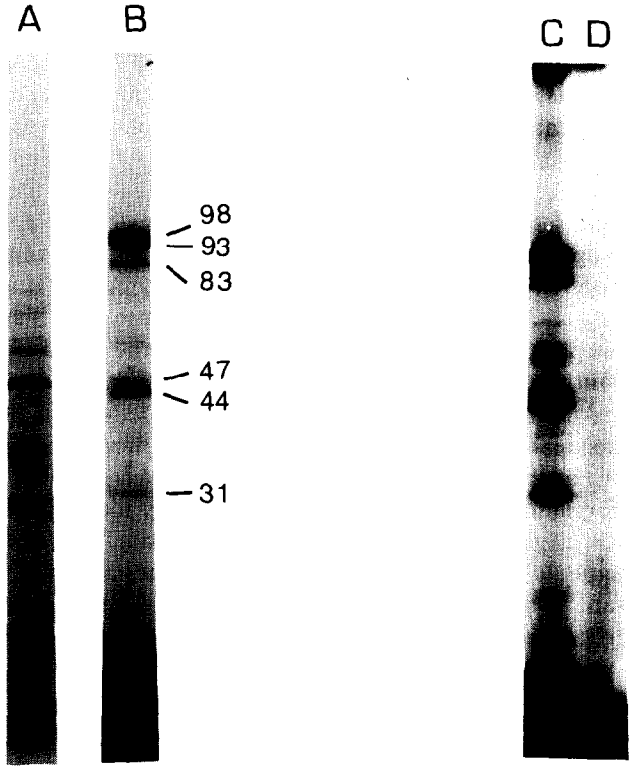

Fig. 1. Polypeptide phosphorylation in the plasma membrane of *A. nidulans* cells. Polypeptide analysis by SDS-PAGE of plasma membrane prepared from nitrate-grown cells, after 30 min incubation with [γ-^{32}P]ATP and Mg^{2+}. Coomassie blue staining (lane A) and autoradiograph (lane B) of the same sample. Overexposed autoradiographs of SDS-PAGE gels of plasma membranes incubated with [γ-^{32}P]ATP (lane C) or [α-^{32}P]ATP (lane D). Figures next to lane B indicate the M_r determined for the phosphopeptides.

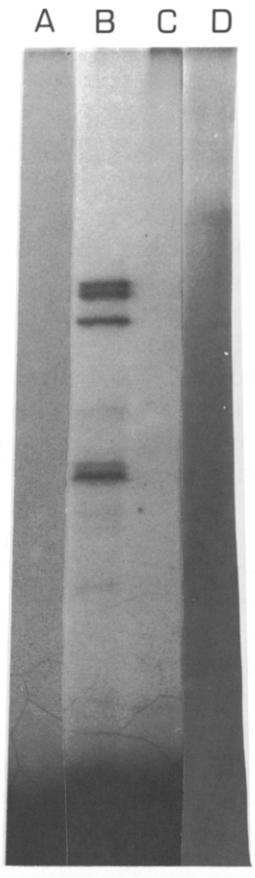

Fig. 2. Temperature sensitivity and Mg^{2+}-requirement of the plasma membrane protein kinase activity of *A. nidulans*. Autoradiographs of SDS-PAGE gels of boiled (lane A) and non-boiled (lane B) plasma membranes incubated with [γ-^{32}P]ATP and 5 mM Mg^{2+}. In lanes C and D, non-boiled membranes were incubated with [γ-^{32}P]ATP and 5 mM Ca^{2+} or no divalent cations, respectively.

Fig. 1 (lanes C and D) shows that polypeptide labelling was only observed after incubation with [γ-^{32}P]ATP, no radioactive bands being detected after incubation with [α-^{32}P]ATP even on overexposed films. This indicates that the observed polypeptide labelling is exclusively due to incorporation of the radioactive phosphate in the γ-position, i.e. to protein phosphorylation.

When the cytoplasmic membrane preparations were boiled for 5 min prior to incubation with [γ-^{32}P]ATP, the protein phosphorylation capacity was lost (Fig. 2), although the polypeptide profile observed after Coomassie blue staining was identical to that of non-boiled membranes (not shown). When Mg^{2+} was not present in the incubation mixture or it was replaced by Ca^{2+}, no protein phosphorylation was observed (Fig. 2), although, again, the polypeptide profile was unchanged (not shown).

These results indicate that in the cytoplasmic membranes of nitrate-grown *A. nidulans* cells exists a temperature-sensitive Mg^{2+}-dependent protein kinase activity able to catalyze in vitro the specific phosphorylation by ATP of these polypeptides of 98, 93, 83, 47, 44, and 31 kDa.

3.2. *Sensitivity of the plasma membrane protein kinase activity to in vivo NH_4^+ exposure*

Cytoplasmic membranes were prepared from nitrate-grown *Anacystis* cells which had been subjected to ammonium exposure during 5 min prior to cell disruption, and their protein kinase activity was tested. Fig. 3 shows that the polypeptide pattern of the cytoplasmic membranes from cells briefly exposed to ammonium was similar to that of control cells, not exposed to ammonium; however, phosphorylated polypeptides were virtually undetectable in the membranes from ammonium-exposed cells. This lack of protein phosphorylation capacity suggests that the plasma membrane protein kinase activity is sensitive to in vivo exposure of the cells to ammonium. The short time of the treatment precludes an effect of ammonium on protein synthesis, as corroborated by the similarity of the polypeptide profiles in Fig. 3.

3.3. *Alkaline phosphatase activity in the soluble fraction*

Alkaline phosphatase activity was assayed in both the cytoplasmic membrane and the soluble fractions of nitrate-grown cells exposed and non-exposed to ammonium. Virtually no alkaline phosphatase activity was detected in the cytoplasmic membrane preparations of

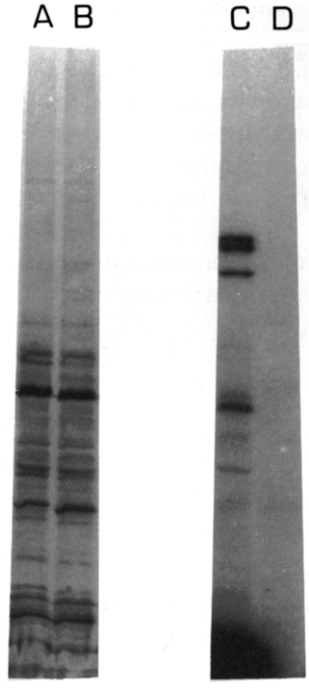

Fig. 3. Effect of in vivo exposure of *A. nidulans* cells to ammonium on the plasma membrane protein kinase activity. Coomassie blue stained SDS-PAGE polypeptide profiles of plasma membranes prepared from control nitrate-grown *A. nidulans* cells (lane A) and from cells subjected to 5 min of in vivo exposure to 5 mM NH_4Cl (lane B). Autoradiographs of the same gels (lanes C and D, respectively).

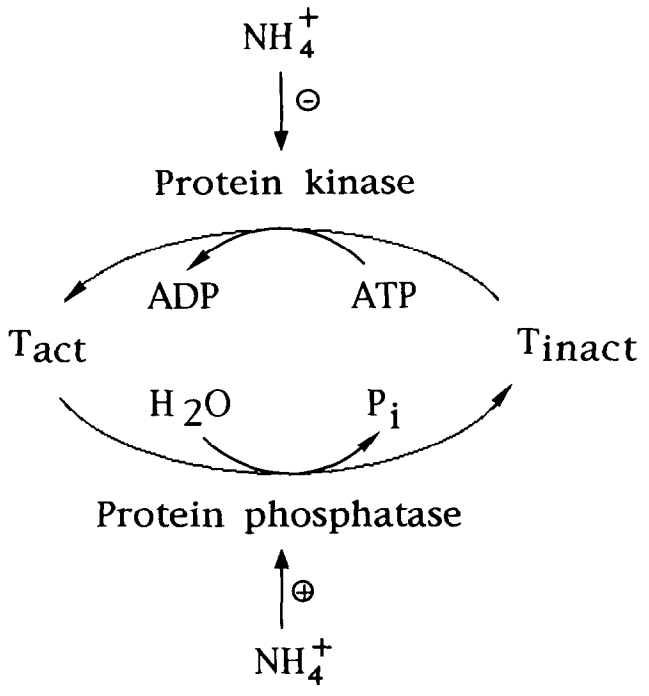

Fig. 4. Proposed model for the regulation of nitrate transport activity by protein phosphorylation/dephosphorylation. Explanation in the text.

either type of cells (not shown), but it was readily detected in the soluble fractions [24]. As Table 1 shows, the alkaline phosphatase activity of the soluble fraction of cells exposed to ammonium was two-fold higher than that of control cells not exposed to ammonium. Interestingly, the increase in alkaline phosphatase activity elicited by ammonium was accompanied by a change in its $K_{m(pNPP)}$ value, which was half of that determined in the soluble fraction of control cells. Increases in alkaline phosphatase activity in response to phosphate deprivation have been reported in *Anacystis nidulans* [24–26] but, to the best of our knowledge, no such changes have been previously reported in response to nitrogen sources.

4. Discussion

The results presented above show that the cytoplasmic membranes of nitrate-grown *A. nidulans* cells contain a Mg^{2+}-dependent protein kinase activity able to phosphorylate in vitro several plasma membrane polypeptides including the 47-kDa protein involved in nitrate transport. In response to in vivo ammonium exposure for 5 min, the protein kinase activity functional in the plasma membrane of nitrate-grown cells would be completely inhibited and, in parallel, an alkaline phosphatase activity of the soluble fraction would be activated more than two-fold, increasing also its affinity for the artificial substrate *p*NPP. These findings can be related to the decrease in the phosphorylation status of the P_{II} protein elicited by ammonium [8] and to the inhibition by ammonium of nitrate transport [9,15]. As reported in [8], P_{II} is phosphorylated in nitrate-grown cells, and loses most labelling after 5 min of exposure of the cells to ammonium. The protein kinase and phosphatase system catalyzing the reversible modification of P_{II} should have a similar responsiveness to ammonium than the plasma membrane protein kinase and soluble phosphatase reported here. Also, upon exposure to ammonium of nitrate-grown cells, nitrate transport activity is completely inhibited [9,15]. A mechanism of reversible covalent modification by protein phosphorylation/dephosphorylation might be involved in the ammonium inhibition of nitrate transport. The results presented above are consistent with a model of metabolic interconversion (Fig. 4) in which a plasma membrane protein kinase activity would phosphorylate some components of the nitrate transporter, such as the 47-kDa polypeptide, and/or a regulatory protein, maintaining the nitrate transporter in its active form. A soluble phosphatase activity would catalyze the corresponding dephosphorylation rendering the inactive form. In the absence of ammonium, with an active protein kinase and a low phosphatase activity, the nitrate transporter would be mainly in its active form. In response to ammonium addition, which triggers a signal transduction chain, the protein kinase would be inhibited and the phosphatase activated, resulting in inactivation of the nitrate transporter.

The actual mechanism involved in the ammonium-induced inhibition of nitrate transport might be more complex than the hypothesis summarized in Fig. 4. Intracellular levels of effectors, resulting from the relative metabolite fluxes of ammonium assimilation and CO_2 fixation [27], would possibly contribute to the final multimodulation of the system. The regulatory model, which is being currently tested in our laboratory, would allow the rapid, effective and reversible responses to ammonium experimentally verified for nitrate transport in *Anacystis*.

Table 1
Effect of in vivo ammonium exposure on alkaline phosphatase activity in the soluble fraction of nitrate-grown *A. nidulans* cells

Treatment of cells	Alkaline phosphatase	
	V_{max} (mU/mg protein)	K_m (*p*NPP) (mM)
None	1.3 ± 0.1	2.6 ± 0.4
5 mM NH_4Cl, 5 min	2.7 ± 0.0	1.2 ± 0.1

Data are mean ± S.E.M. of the kinetic parameters. Ammonium exposure of the cells was performed as described in section 2.

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