Nitrogen source-dependent expression of a 126 kDa protein in the plasma membrane of the cyanobacterium *Synechococcus* PCC 7942

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Abstract The expression of a 126 kDa protein in the cytoplasmic membrane of *Synechococcus* PCC 7942 is shown to be dependent on the nitrogen source. It is absent in ammonium-grown cells and its quantity is inversely related to the concentration of nitrate or nitrite in the growth medium. Addition of ammonium-grown cells to a medium containing nitrate or L-methionine-DL-sulfoximine results in the expression of this protein. It is present in the plasmalemma of the *Synechococcus* NC3 mutant (*nrt*C gene deleted) and absent in the NA3 mutant (*nrt*ABCD genes deleted). These results may suggest involvement of the 126 kDa protein in nitrate transport through *Synechococcus* cytoplasmic membrane.

Key words: Nitrogen source; Plasma membrane protein; Adaptation; Nitrate transport; *Synechococcus*

1. Introduction

Nitrate is the major source of nitrogen for photosynthetic organisms including cyanobacteria, in particular non-N_2-fixing strains [1–3]. The first and rate-limiting step [4] of nitrate assimilation is its transport into the cell. The nitrate uptake takes place through an active multicomponent transport system [1] in the plasma membrane that exhibits a high affinity for nitrate [3,5] and nitrite [6]. When nitrate-grown cells are exposed to ammonium, the nitrate transport is completely inhibited and the protein composition of the cytoplasmic membrane is modified [7–9]. In the unicellular non-nitrogen-fixing cyanobacterium *Synechococcus* PCC 7942 the four proteins required for active transport of nitrate are encoded by the genes *nrt*ABCD [10,11]. These four genes are clustered with the genes *nar*B and *nir*A encoding proteins involved in the nitrate reduction to ammonium and form the *nir*A-*nrt*ABCD-*nar*B operon (the *nir*A operon). The nitrate transport system of *Synechococcus* PCC 7942 translocates nitrite as well as nitrate [12–15]. The major cytoplasmic membrane protein of nitrate-grown cells [8] is the 45 kDa substrate-binding lipoprotein encoded by *nrt*A [15]. This protein is absent in the cytoplasmic membrane of ammonium-grown cells [7–9].

In this paper, we shall report on variations in the content of a 126 kDa protein of the *Synechococcus* PCC 7942 cytoplasmic membrane, depending on amounts of nitrate and nitrite in the growth medium. Expression of this protein was inhibited by ammonium.

2. Materials and methods

2.1. Strains and growth conditions

The *Synechococcus* PCC 7942 wild-type cells and the mutants NA3 and NC3 (gift of Prof. T. Omata) were grown at 30°C in sterilized liquid media, under gentle stirring and bubbling with 2% CO_2-enriched air and illuminated by 50 μE m^{-2} s^{-1} white light. The basal medium used was a nitrogen-free medium obtained by modification of Van Allen medium [16] as previously described [17]. Ammonium-containing medium was prepared by addition of 5 mM NH_4Cl to the basal medium. Nitrate-containing medium was prepared by addition of $NaNO_3$ or KNO_3 at different concentrations in the presence or in the absence of NaCl or KCl in the basal medium. Nitrite-containing medium was prepared by addition of 2 mM or 15 mM $NaNO_2$. For change of nitrogen source, the pelleted cells (5000 × *g*, 12 min at 25°C) were transferred and incubated under the selected growth conditions. Prior to harvesting the non-contamination of each flask of culture was checked on minimum medium agar plates incubated in the dark.

2.2. Membrane preparation and characterization

The preparation and purification of the cytoplasmic membrane vesicles and the protein determination of the purified membranes were performed using the techniques already described [17,18]. The polypeptide composition of the cytoplasmic membrane preparations were analyzed by SDS-PAGE. The cytoplasmic membranes were precipitated with trichloroacetic acid (TCA) (10% final concentration) and the pellet was resuspended in 100 mM Tris, pH 8.5, containing 2.5% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 0.01% (w/v) bromophenol blue, and incubated for 15 h at 30°C. The samples were centrifuged for 5 min at 100 000 × *g* in an Airfuge centrifuge from Beckman. The samples were loaded on a 8–25% or 4–15% polyacrylamide gradient gel from Pharmacia or Bio-Rad, respectively. Molecular mass standards from Pharmacia and Bio-Rad were run in each assay. Electrophoresis and staining of protein were performed on a Pharmacia PHAST system or Mini-Protean II electrophoresis cell from Bio-Rad.

2.3. Phosphorylation procedure and electrophoresis

Phosphorylation by [γ-^{32}P]ATP was performed with membranes from nitrate- and ammonium-grown cells using the procedure already described [17,19]. Briefly, after 1 min incubation of two batches of each membrane preparation (100 μg protein) with 0.09 mM [γ-^{32}P]ATP (110 GBq/mmol, Amersham) in the presence of $MgSO_4$ ($[Mg^{2+}]/[ATP] = 2.5$), the reaction was stopped by TCA addition (10% final concentration) and after centrifugation for 5 min at 13 000 × *g* (Centrifuge 5415 from Eppendorf) the pellets were resuspended in HEPES 10 mM pH 7.4 containing unlabeled ATP (10 times more concentrated than the labeled ATP) and $MgSO_4$ ($[Mg^{2+}]/[ATP] = 2.5$) to avoid non-specific binding. After a 2 min incubation, proteins were precipitated with TCA and the pellets were resuspended in denaturing solution either at pH 8.5 (SDS-PAGE, alkaline conditions) or at pH 4 (TDAB-PAGE, acid conditions). The two PAGEs (10% acrylamide gel) were run simultaneously in two Mini-Protean II electrophoresis cells from Bio-Rad. The gels were Coomassie stained and dried. After 72 h exposure at −80°C, autoradiograms were obtained on Biomax MS film from Kodak.

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Abbreviations: MSX, L-methionine-DL-sulfoximine; TCA, trichloroacetic acid; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TDAB-PAGE, tetradecyltrimethylammonium bromide-polyacrylamide gel electrophoresis

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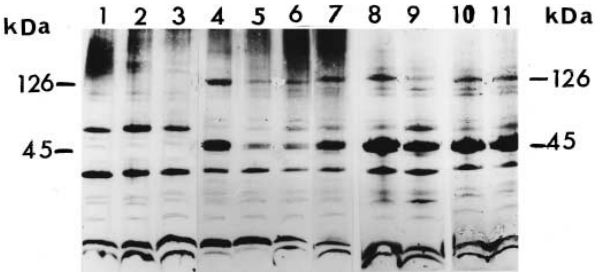

Fig. 1. Influence of the nitrogen source on the polypeptide composition of cytoplasmic membranes from *Synechococcus* PCC 7942. Cytoplasmic membranes were prepared from nitrate-, nitrite- and ammonium-grown cells. Samples containing 3 μg (lanes 1–7) and 4 μg (lanes 8–11) of protein were loaded on an 8–25% polyacrylamide gradient gel and stained with $AgNO_3$ using a Phast System (Pharmacia). Cytoplasmic membranes from 5 mM NH_4Cl-grown cells in the presence of 173 mM KCl (lane 1) and 173 mM NaCl (lane 2); from 5 mM NH_4Cl-grown cells (lane 3); from 2 mM and 175 mM $NaNO_3$-grown cells (lanes 4, 5); from 15 mM and 2 mM $NaNO_2$-grown cells (lanes 6, 7); from 2 mM and 175 mM KNO_3-grown cells (lanes 8, 9); from 2 mM $NaNO_3$-grown cells in the presence of 173 mM KCl (lane 10) and 173 mM NaCl (lane 11).

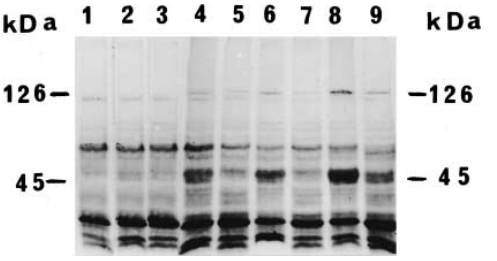

Fig. 2. Time-dependent modification of the 126 kDa and 45 kDa plasmalemma protein amounts upon transfer of ammonium-grown *Synechococcus* PCC 7942 cells to nitrate media. The 5 mM NH_4Cl-grown cells (lane 1) were transferred to a medium containing 2 mM or 175 mM $NaNO_3$ for 3 h (lanes 2 and 3), 6 h (lanes 4 and 5), 12 h (lanes 6 and 7) and 18 h (lanes 8 and 9). After these times, cytoplasmic membranes were prepared and samples containing 100 μg of protein were subjected to SDS-PAGE on a 10% polyacrylamide gel. The protein detection was performed by Coomassie staining.

3. Results

3.1. *Effect of different nitrogen sources on the polypeptide composition of the cytoplasmic membrane*

The polypeptide composition of cytoplasmic membranes from ammonium-, nitrate- and nitrite-grown *Synechococcus* PCC 7942 cells was analyzed by SDS-PAGE. In addition to the variation of the 45 kDa protein already described [7–9], a 126 kDa protein was found to vary in cytoplasmic membrane depending on the nitrogen source used (Fig. 1). The amount of the 126 kDa as well as the 45 kDa band was inversely related to the nitrate concentration in the medium. The cells grown in the presence of the highest concentration of nitrate (175 mM) exhibited the smallest amount of these bands (Fig. 1, lane 5). Conversely, the cells grown in the presence of the lowest concentration of nitrate (2 mM) presented the highest intensities (Fig. 1, lane 4). The same effect was noted for the high (15 mM) and the low (2 mM) concentrations of nitrite (Fig. 1, lanes 6 and 7, respectively). The 126 kDa protein was not present in the thylakoid membrane whatever the concentration of nitrate or nitrite in the growth medium (data not shown).

The described change of the polypeptide profile was independent of the Na^+/K^+ variation. The cells grown in the presence of the same nitrate concentration (2 mM) but with various Na^+/K^+ ratios had the same amounts of the 126 kDa as well as the 45 kDa proteins (Fig. 1, lanes 8, 10 and 11). At the same time, in the membranes from the cells grown with the same Na^+/K^+ ratio, the 126 kDa and 45 kDa protein amounts presented the same inverse relationship to the nitrate concentration (Fig. 1, lanes 9, 10 and 5, 11, respectively). Both proteins were absent in the ammonium-grown cells whatever the Na^+/K^+ variation in the medium (Fig. 1, lanes 1–3).

3.2. *Time-dependent modification of the cytoplasmic membrane polypeptide composition in response to change in the nitrogen source*

Transfer of ammonium-grown cells to a medium containing nitrate as the sole nitrogen source induced the appearance not only of the 45 kDa protein [8] but also the 126 kDa protein (Fig. 2). A progressive increase of the 45 kDa protein was observed after 3 h in the presence of 5 mM nitrate [8]. The 126 kDa protein was not observable at this time (Fig. 2, lanes 2 and 3), perhaps due to its small amount. Taking into account that the major (about 25%) cytoplasmic membrane protein of 5 mM nitrate-grown cells is the 45 kDa protein [8], the amount of the 126 kDa protein was estimated to be 2.5% and 1% of the membrane proteins from 2 mM and 175 mM nitrate-grown cells, respectively (data not shown). After 6 h, in the presence of nitrate, a progressive increase in the 126 kDa protein was observed (Fig. 2, lanes 4 and 5). Fig. 2 shows that the amount of the 126 kDa protein in the cytoplasmic membrane as well as that of the 45 kDa protein was inversely related to the nitrate concentration in the growth medium. Up to 12 h, this difference was clearer (Fig. 2, lane 6 and 7). Finally, after 18 h the plasmalemma protein compositions from 2 mM and 175 mM nitrate-grown cells were similar to those observed on adapted cells (Fig. 2, lanes 8, 9). The same time dependence was noted on the 2 mM and 15 mM nitrite-grown cells (data not shown).

The inverse transfer of the 2 mM and 175 mM nitrate-grown cells to the ammonium-containing medium was also

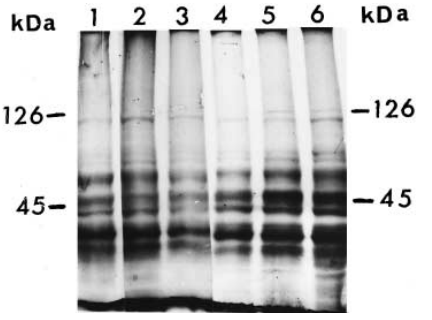

Fig. 3. Effect of MSX on the time-dependent expression of the 126 kDa and 45 kDa plasmalemma proteins from ammonium-grown cells. The 5 mM NH_4Cl-grown cells (lane 1) were treated with 100 μM MSX in the presence of 2 mM or 175 mM $NaNO_3$ for 3 h (lanes 2 and 3), 6 h (lanes 5 and 6) and 6 h in the absence of $NaNO_3$ (lane 4). After the treatment, cytoplasmic membranes were prepared and samples containing 100 μg of protein were subjected to SDS-PAGE on a 4–15% polyacrylamide gradient gel (Bio-Rad). The protein detection was performed by Coomassie staining.

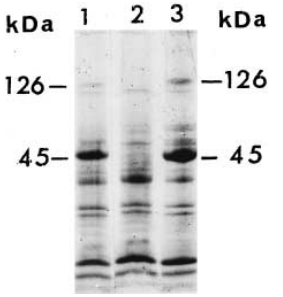

Fig. 4. The polypeptide composition of the cytoplasmic membrane from NC3 and NA3 mutants. Cytoplasmic membranes were prepared from 60 mM $NaNO_3$-grown cells of NC3 (lane 1), NA3 (lane 2) and from 15 mM $NaNO_3$-grown cells of NC3 (lane 3). Samples containing 4 μg of protein were loaded on an 8–25% polyacrylamide gradient gel and stained with Coomassie using a Phast System (Pharmacia).

examined. The 126 kDa protein and the 45 kDa protein vanished after 18 h (data not shown).

3.3. *Time-dependent modification of the cytoplasmic membrane polypeptide composition in response to L-methionine-DL-sulfoximine (MSX) treatment of the ammonium grown cells*

MSX effectively inhibits ammonium assimilation through inactivation of glutamine synthetase [20]. Treatment of ammonium-grown cells with 100 μM MSX and nitrate induced the appearance of the 126 kDa protein as well as the 45 kDa protein (Fig. 3). Presence of the 126 kDa protein was observed only up to 6 h growth. However, the plasmalemma protein compositions from cells grown with 2 mM and 175 mM nitrate were almost similar (Fig. 3, lanes 5 and 6). In the control experiment, i.e. growth in the presence of 100 μM MSX but without nitrate, the appearance of the 126 kDa protein was not induced at 6 h (Fig. 3, lane 4). Under these conditions, the plasmalemma protein composition was similar to that observed on ammonium-grown cells for 3 h in the presence of nitrate and 100 μM MSX (Fig. 3, lanes 2 and 3).

3.4. *The polypeptide composition of the cytoplasmic membranes from NC3 and NA3 mutant cells*

In the *Synechococcus* NC3 mutant (*nrt*C gene deleted, personal communication), the 126 kDa protein was also expressed in the plasmalemma of nitrate-grown cells (Fig. 4, lane 1), whereas under the same conditions it was absent in the NA3 mutant (*nrt*ABCD genes deleted) [15] (Fig. 4, lane 2). The NC3 mutant cells grown in the presence of different concentrations of nitrate presented the same inverse dependence of the 126 and the 45 kDa protein amounts on nitrate concentration (Fig. 4, lanes 1 and 3).

3.5. *Phosphorylation of cytoplasmic membrane proteins from ammonium- and nitrate-grown cells by [γ^{32}P]ATP*

Previously we have described the [γ-^{32}P]ATP labeling of plasmalemma proteins from nitrate-grown cells under alkaline and acid conditions [17]. We observed that the same proteins were labeled in the cytoplasmic membranes from ammonium-grown cells, i.e. 82, 74, 53 and 42 kDa under alkaline conditions and 110, 90 and 71 kDa under acid conditions. However, the labeling was less intense for the 82 kDa and more intense for the 74 and 42 kDa proteins of the plasmalemma from ammonium-grown cells. Under both pH conditions the 126 kDa protein and the 45 kDa protein were not phosphorylated. The overall phosphorylation was higher in the membranes from ammonium-grown cells than nitrate-grown ones.

4. Discussion

It is well known that the presence in the cytoplasmic membrane from *Synechococcus* of the 45 kDa substrate binding protein [15] depends on the nitrogen source [7–9]. We found that the presence of one more protein with an apparent molecular mass of 126 kDa in the cytoplasmic membrane of *Synechococcus* PCC 7942 is also dependent on the nitrogen source. Indeed, this protein was absent in the cytoplasmic membrane from ammonium-grown cells. The quantity of the 126 kDa protein was inversely related to the concentration of nitrate or nitrite in the growth medium. This behavior suggests that this protein could be involved in nitrate and nitrite transport. This observation is related to the assumption that the same system operates the active transport of both substrates [12–15].

The absence of the 126 kDa protein in the cytoplasmic membrane from ammonium-grown cells could be correlated with the repression of the *nir*A operon transcription [1,21,22] by ammonium or its metabolites generated internally which give rise to an effective inhibition of nitrate transport activity [23,24]. We have observed that transfer for 6 h of ammonium-grown cells to a medium containing nitrate, as the sole nitrogen source, resulted in the expression of the 126 kDa protein in an amount inversely correlated with the nitrate concentration. The negative effect of ammonium on nitrate transport is abolished by MSX which inhibits glutamine synthetase [20]. In the presence of this inhibitor and nitrate, the existence of this protein was noted on cells grown for 6 h. It should be emphasized that without nitrate the 126 kDa protein did not appear at this time. The same MSX effect was observed for the 45 kDa protein.

Mutants affected in the genes encoding the different components of the nitrate/nitrite transport system are very informative. Indeed, mutant NA3, constructed by deleting the *nrt*ABCD genes [15], had no protein at the 126 kDa level in the presence of nitrate whereas the *Synechococcus* NC3 mutant, with the entire *nrt*C gene deleted, expressed this protein with the same dependence on the nitrate concentration as the wild type. These observations suggest the involvement of the 126 kDa protein in the nitrate transport system.

If the nitrate transport is controlled by a protein phosphorylation-dephosphorylation mechanism [25], it involves proteins other than the 45 kDa and 126 kDa proteins. Indeed, the phosphorylation of the 126 kDa and 45 kDa proteins was not observed under our conditions. This is at variance with result of Rodriguez et al. who saw some 45 kDa protein phosphorylation [25]. The phosphorylation of the 110 kDa protein corresponds to the P-type ATPase as previously shown [17] and it is interesting to note that this ATPase was equally represented in the plasmalemma from the nitrate- and ammonium-grown cells.

The 126 kDa protein gives rise to important questions about the expression, structure and function of this protein in the nitrate transport.

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References

[1] Flores, E. and Herrero, A. (1994) in: The Molecular Biology of Cyanobacteria (Bryant, D.A., Ed.), Vol. 1, pp. 487–517, Kluwer, Dordrecht.
[2] Guerrero, M.G., Vega, J.M. and Losada, M. (1981) Annu. Rev. Plant Physiol. 32, 169–204.
[3] Guerrero, M.G. and Lara, C. (1987) in: The Cyanobacteria (Fay, P. and Van Baalen, R., Eds.), pp. 163–186, Elsevier, Amsterdam.
[4] Lara, C., Rodriguez, R. and Guerrero, M.G. (1993) J. Physiol. 29, 389–395.
[5] Flores, E., Guerrero, M.G. and Losada, M. (1983) Biochem. Biophys. Acta 722, 408–416.
[6] Flores, E., Herrero, A. and Guerrero, M.G. (1987) Biochem. Biophys. Acta 896, 103–108.
[7] Omata, T., Ohmori, M., Arai, N. and Ogawa, T. (1989) Proc. Natl. Acad. Sci. USA 86, 6612–6616.
[8] Sivak, M.N., Lara, C., Romero, J.M., Rodriguez, R. and Guerrero, M.G. (1989) Biochem. Biophys. Res. Commun. 158, 257–262.
[9] Madueno, F., Vega-Palas, M.A., Flores, E. and Herrero, A. (1988) FEBS Lett. 239, 289–291.
[10] Omata, T., Andriesse, X. and Hirano, A. (1993) Mol. Gen. Genet. 236, 193–202.
[11] Luque, I., Herrero, A., Flores, E. and Madueno, F. (1992) Mol. Gen. Genet. 232, 7–11.
[12] Madueno, F., Flores, E. and Guerrero, M.G. (1987) Biochem. Biophys. Acta 896, 109–112.
[13] Rodriguez, R., Lara, C. and Guerrero, M.G. (1992) Biochem. J. 282, 639–643.
[14] Luque, I., Flores, E. and Herrero, A. (1994) Biochem. Biophys. Acta 1184, 296–298.
[15] Maeda, S.-I. and Omata, T. (1997) J. Biol. Chem. 272, 3036–3041.
[16] Van Allen, R. (1968) J. Physiol. 4, 1–4.
[17] Fresneau, C., Rivière, M.-E. and Arrio, B. (1993) Arch. Biochem. Biophys. 306, 254–260.
[18] Omata, T. and Ogawa, T. (1986) Plant Physiol. 80, 525–530.
[19] Amory, A., Foury, F. and Goffeau, A. (1980) J. Biol. Chem. 255, 9353–9357.
[20] Flores, E., Romero, J.M., Guerrero, M.G. and Losada, M. (1983) Biochim. Biophys. Acta 725, 529–532.
[21] Suzuki, I., Sugiyama, T. and Omata, T. (1993) Plant Cell Physiol. 34, 1311–1320.
[22] Kikuchi, H., Aichi, M., Suzuki, I. and Omata, T. (1996) J. Bacteriol. 178, 5822–5825.
[23] Lara, C., Romero, J.M. and Guerrero, M.G. (1987) J. Bacteriol. 169, 4376–4378.
[24] Lara, C., Rodriguez, R. and Guerrero, M.G. (1993) Physiol. Plant. 89, 582–587.
[25] Rodriguez, R., Garcia-Gonzalez, M., Guerrero, M.G. and Lara, C. (1994) FEBS Lett. 350, 19–23.