Vol. 158, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
January 16, 1989 Pages 257-262

RELATIONSHIP BETWEEN A 47-kDa CYTOPLASMIC MEMBRANE POLYPEPTIDE AND NITRATE TRANSPORT IN *Anacystis nidulans*

Mirta N. Sivak[1], Catalina Lara, José M. Romero, Rocío Rodríguez and Miguel G. Guerrero[2]

Instituto de Bioquímica Vegetal y Fotosíntesis, Universidad de Sevilla-Consejo Superior de Investigaciones Científicas, Apartado 1113, 41080 Sevilla, Spain

Received November 8, 1988

The polypeptide composition of cytoplasmic membranes of the cyanobacterium *Anacystis nidulans* changes in response to variations in the nitrogen source available to the cells, differing specifically in the amount of a polypeptide of 47-kDa molecular mass. Synthesis of the polypeptide and expression of nitrate transport activity are repressed by ammonium. Transfer of ammonium-grown cells to a medium containing nitrate as the sole nitrogen source results in parallel development of the 47-kDa polypeptide and nitrate transport activity of the cells. These results suggest the involvement of the 47-kDa cytoplasmic membrane polypeptide in nitrate transport by *A. nidulans*.

The entrance of nitrate into the cell is the initial step in nitrate assimilation, followed by nitrate reduction to ammonium and by subsequent ammonium incorporation to carbon skeletons yielding amino acids. Although there is a general belief in the involvement of a nitrate transport system in nitrate utilization, very little is known of the biochemistry of nitrate transport (1-5). Recently, intracellular accumulation of

[1]Visiting scientist under Spain-UK Acción Integrada no. 60/37. Permanent address: Research Institute for Photosynthesis, The University of Sheffield, Sheffield S10 2TN, U.K.

[2]To whom correspondence and reprint requests should be addressed.

Abbreviation: Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl) ethyl]-glycine.

0006-291X/89 $1.50

nitrate, indicative of the operation of an energy-requiring nitrate transport system, has been measured in intact cells of the cyanobacterium Anacystis nidulans. The system was shown to be sensitive to the regulation exerted by products of both ammonium and CO_2 assimilation, supporting the contention that nitrate assimilation in cyanobacteria is tightly controlled at the level of substrate supply to the cell (6).

Particular polypeptides present in the cytoplasmic membrane of Anacystis are preferentially synthesized upon adaptation of cells to low CO_2 (7) or sulfur deprivation (8), and their involvement in the transport of inorganic carbon and sulfate has been proposed. We report here results showing marked changes in the level of a polypeptide of 47-kDa present in purified cytoplasmic membranes of A. nidulans cells in response to differences in the nature of the nitrogen source available to the cells. A comparison has been made between the abundance of the 47-kDa polypeptide and the capacity of cells to accumulate nitrate. An involvement of this 47-kDa cytoplasmic membrane polypeptide in nitrate transport is inferred from the data.

MATERIALS AND METHODS

Anacystis nidulans (Synecochoccus leopoliensis 1402-1, from Göttingen University, Göttingen, Germany) was grown photoautotrophically with either nitrate or ammonium as the nitrogen source as described previously (9) to a cell density equivalent to 15-20 μg chlorophyll ml^{-1}. Cells were harvested by centrifugation (8000 g x 10 min at room temperature). For any change of medium, the pelleted cells were washed with and resuspended in the required medium to a cell concentration equivalent to 15-20 μg chlorophyll ml^{-1}.

Cytoplasmic and thylakoid membranes were prepared according to the procedure of Omata and Ogawa (7), except that the concentration of EDTA during the lysozyme treatment of cells was 0.5 mM. SDS-polyacrilamide gel electrophoresis of the membrane preparations was performed in gel slabs according to Laemmli (10). Gels were stained with Coomasie Brilliant Blue. Densitometry of stained gels was carried out by measuring absorbance at 560 nm using a Desaga CD 60 densitometer.

Nitrate transport activity was determined by measuring intracellular nitrate by ion-exchange high-pressure liquid chromatography in acid lysates of cells subject to silicone oil centrifugation (6). The assay medium contained 25 mM Tricine/NaOH/KOH buffer (pH 8.3), 10 mM $NaHCO_3$, 50 μM KNO_3 and an amount of cells equivalent to 30 μg chlorophyll ml^{-1}. Assays, performed at 40°C in the dark with continuous shaking, were initiated by nitrate addition and stopped after 6 min by rapid centrifugation of the cells through silicone oil (6).

Protein was determined by the method of Bradford (11). Chlorophyll a was determined after methanol extraction according to (12).

RESULTS AND DISCUSSION

The polypeptide composition of purified cytoplasmic membranes has been examined in A. nidulans cells grown in media containing either nitrate or ammonium as the nitrogen source (Fig. 1). The polypeptide profile of nitrate-grown cells, able to accumulate nitrate at intracellular levels of about 200 μM, is analogous to those reported by other workers for cytoplasmic membranes of A. nidulans cells which were routinously grown on standard media containing nitrate as the sole source of nitrogen (7-8). A polypeptide of 47-kDa molecular mass, representing about 25% of total cytoplasmic membrane protein in nitrate-grown cells, was however absent or present at very low levels (below 2% total plasma membrane protein) in ammonium-grown cells (Fig. 1), which were otherwise unable to accumulate nitrate. Presence of the 47-kDa polypeptide is specific of cytoplasmic membranes. There was a negligible amount of the polypeptide in thylakoid membranes preparations of nitrate-grown cells (data not shown), that can be ascribed to a small contamination of this fraction with cytoplasmic membranes (7).

Changes in the level of the 47-kDa cytoplasmic membrane polypeptide and nitrate transport activity upon transfer of ammonium-grown A. nidulans cells to media containing different nitrogen sources were examined. Significant amounts of the 47-kDa polypeptide were found in the absence of ammonium, i.e. in medium containing nitrate or no available nitrogen source (Fig. 2), with concomitant capacity of the cells to accumulate intracellular nitrate. Actually, the cells transferred to medium lacking a nitrogen source exhibited levels of the 47-kDa polypeptide about 50% higher and accumulated about 50% more nitrate than those transferred to nitrate medium (data not shown). The presence of ammonium in the medium always led to negligible levels of the 47-kDa polypeptide (and nitrate transport activity) even if nitrate was simultaneously present (Fig. 2). These results indicate that the negative effect exerted by ammonium on the development of the nitrate transport system and the 47-kDa cytoplasmic membrane polypeptide cannot be overriden by nitrate. Appearence of both the 47-kDa polypeptide and nitrate transport capacity upon transfer of ammonium-grown cells to nitrate medium was prevented by 50 μg/ml chloramphenicol (data not shown). It would thus appear that regulation of

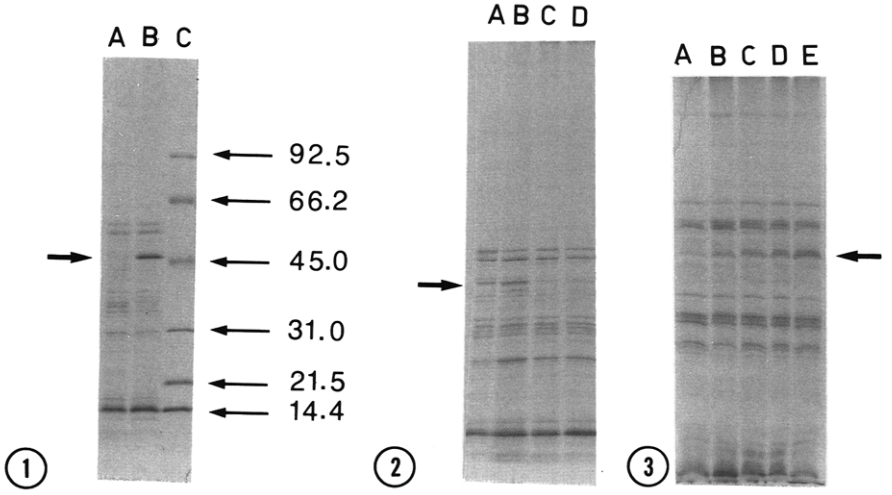

Figure 1. Polypeptide composition of purified cytoplasmic membranes of Anacystis nidulans. Cytoplasmic membranes were prepared from cells grown on media containing ammonium (A) or nitrate (B) as the sole nitrogen source. Aliquots containing each 20 g protein were subjected to electrophoresis in 7.5-15% polyacrilamide-SDS slab gels. Molecular mass standars (C) were: phosphorylase b, 92.5 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; bovine carbonic anhidrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa, and lysozyme, 14.4 kDa. The arrow in the left indicates the 47-kDa polypeptide.

Figure 2. Influence of the nitrogen source on the polypeptide composition of cytoplasmic membranes of A. nidulans. Ammonium--grown cells were transferred to media containing 5 mM KNO_3 (A); no nitrogen source (B); 5 mM NH_4NO_3 (C); and 2.5 mM $(NH_4)_2SO_4$ (D). After 5 h, cytoplasmic membranes were prepared from each batch of cells and aliquots containing each 30 μg protein were subjected to electroforesis in 7.5-15% polyacrilamide-SDS slab gels. The arrow in the left indicates the 47-kDa polypeptide.

Figure 3. Development of the 47-kDa polypeptide upon transfer of ammonium-grown A. nidulans cells to nitrate medium. Ammonium--grown cells (A) were transferred to a medium containing 5 mM KNO_3 for 1.5 h (B); 3.0 h (C); 4.5 h (D); and 6.0 h (E). After these times, cytoplasmic membranes were prepared and aliquots containing 30 (A); 35 (B); 32 (C); 25 (D); and 26 (E) μg protein were subjected to electroforesis in 10% polyacrilamide-SDS slab gels. The arrow in the right indicates the 47-kDa polypeptide.

synthesis of this polypeptide, and of the development of nitrate transport activity, is exerted through repression by ammonium (or a product of ammonium metabolism), nitrate being otherwise not required as an inducer. Such a pattern of regulation is analogous to that reported for the enzymes of the nitrate-reducing system of this cyanobacterium (5), namely nitrate reductase (9) and nitrite reductase (13). The 47-kDa cytoplasmic membrane polypeptide might correspond to nitrate or nitrite reductase. Neither enzyme activity was detected, however, in the cytoplasmic membrane preparations. Nitrate reductase activity

was located in the thylakoid fraction, which also exhibited some nitrite reductase activity, most of the latter appearing in the soluble fraction. Moreover, estimates of the subunit molecular mass for nitrate and nitrite reductases in A. nidulans are above 47 kDa, being 75 kDa and 54 kDa, respectively (5). Available evidence thus points to the idea of the 47-kDa polypeptide being a component of the nitrate assimilation system in Anacystis, other than nitrate or nitrite reductases, the synthesis of the latter proteins and of the 47-kDa polypeptide sharing a common regulatory pattern.

The time course of the development of the 47-kDa cytoplamic membrane polypeptide following transfer of ammonium-grown A. nidulans cells to a nitrate medium has been followed. A progresive increase in the level of the 47-kDa polypeptide was manifest after 3 h in the presence of nitrate (Fig. 3). The correlation existing between amount of the 47-kDa polypeptide in the plasma membrane and the capacity of cells to accumulate nitrate becomes evident from the data in Table I, showing the

TABLE I

Increase in the levels of nitrate transport and the 47-kda cytoplasmic membrane polypeptide in Anacystis nidulans following the transfer of ammonium-grown cells to a medium containing nitrate

Time (h)	Intracellular NO_3^- (μM)	47-kDa polypeptide (area units)
0	<2	170
1.5	<2	150
3.0	27	490
4.5	38	570
6.0	76	720
24.0	181	3120

A. nidulans was grown on ammonium-containing medium and at t=0 transferred to medium containing 5 mM KNO_3 as the sole nitrogen source. At the times indicated samples were withdrawn for preparation of cytoplasmic membranes and assays of nitrate transport activity. The abundance of the 47-kDa polypeptide was evaluated by integration of densitometric scanning of stained gel slabs after SDS-polyacrylamide gel electrophoresis of cytoplasmic membrane preparations containing each 30±2 μg of protein. Data are those of a representative experiment.

evolution with time of these two parameters in response to the transfer of cells from ammonium to nitrate medium.

The above results demonstrate the existence in the cytoplasmic membrane of A. nidulans cells of an adaptive 47-kDa polypeptide, the level of which changes in response to the nature of the nitrogen source. Ammonium behaves as a nutritional repressor of the synthesis of this polypeptide, as is also the case for the enzymes of the nitrate-reducing system of this cyanobacterium. This and the correlation found between nitrate transport activity and abundance of the 47-kDa polypeptide strongly suggests the involvement of the latter in the nitrate assimilation system of A. nidulans, most probably as a component of the nitrate transporter.

ACKNOWLEDGEMENTS

Work supported by grant BT 85/45 from Comisión Asesora de Investigación, Spain. We thank Prof. M. Losada for encouragement and support and A. Friend and J. Moreno for skilful technical assistance. The access to some facilities of the Department of Microbiology, University of Sevilla, and Instituto de Recursos Naturales y Agrobiología, CSIC, is much appreciated.

REFERENCES

1. Ullrich, W.R. (1987) In Inorganic Nitrogen Metabolism (Ullrich, W.R., Aparicio, P.J., Syrett, P.J. and Castillo, F., Eds.), pp. 32-38, Springer, Berlin.
2. Beevers L. and Hageman, R.H. (1983) In Inorganic Plant Nutrition, Encyclopedia of Plant Physiology, New Series (Lauchli, A. and Bieleski, R.L., Eds.), Vol. 15A, pp. 351--375, Springer, Berlin.
3. Gibson, J. (1984) Ann. Rev. Microbiol. 38, 135-159.
4. Syrett, P.J. (1981) Can. Bull. Fish. Aquat. Sci. 210, 182-210.
5. Guerrero, M.G. and Lara, C. (1987) In The Cyanobacteria (Fay, P. and Van Baalen, C., Eds.), pp. 163-186, Elsevier, Amsterdam.
6. Lara, C., Romero, J.M. and Guerrero, M.G. (1987) J. Bacteriol. 169, 4376-4378.
7. Omata, T. and Ogawa, T. (1986) Plant Physiol. 80, 525-530.
8. Green, L.S. and Grossman, A.R. (1988) J. Bacteriol. 170, 583-587.
9. Herrero, A., Flores, E. and Guerrero, M.G. (1981) J. Bacteriol. 145, 175-180.
10. Laemmli, U.K. (1970) Nature 227, 680-685.
11. Bradford, M.M. (1976) Anal. Biochem. 72, 248-254.
12. MacKinney, G. (1941) J. Biol. Chem. 160, 315-322.
13. Herrero, A. and Guerrero, M.G. (1986) J. Gen. Microbiol. 132, 2463-2468.