A sensor protein involved in induction of nitrate assimilation in *Azotobacter chroococcum*

María C. Muñoz-Centeno, Ana Peciña, Francisco J. Cejudo, Antonio Paneque*

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

Received 25 June 1996

Abstract **Nitrogen-fixing *Azotobacter chroococcum* cells, but not ammonium- or nitrate-grown cells, exhibited two polypeptide components of 22 and 35 kDa, respectively, that we termed P22 and P35. Bidimensional polyacrylamide gel electrophoresis analysis of preparations from N_2-fixing cells that had been transferred to nitrate medium and then incubated for 2 h revealed that P22 had shifted to a more acidic part of the gel while P35 did not change its electrophoretic pattern. Using [^{32}P]orthophosphoric acid it could be demonstrated that the shift in mobility of P22 was due to the phosphorylation of the polypeptide dependent on nitrate (nitrite). The *A. chroococcum* TR1 strain, which is unable to use nitrate as a nitrogen source and displays activities of nitrogenase, nitrate reductase and nitrite reductase, exhibited both polypeptides. In contrast, P22 and P35 were absent from *A. chroococcum* MCD1, a mutant strain that cannot assimilate nitrate and lacks the nitrate-reducing enzymatic system. The results suggest that P22 could act as a sensor protein for nitrate in *A. chroococcum*.**

Key words: Nitrate assimilation; Sensor protein; *Azotobacter chroococcum*

1. Introduction

Azotobacter chroococcum is a heterotrophic, aerobic bacterium that can use different inorganic nitrogen sources such as ammonia, nitrate, nitrite or dinitrogen. In the nitrate assimilation pathway, three steps can be distinguished, namely, (i) nitrate transport into the cell, which takes place in *A. chroococcum* through a multicomponent system [1]; (ii) reduction of nitrate to nitrite catalyzed by nitrate reductase; and (iii) reduction of nitrite to ammonia catalyzed by nitrite reductase [2], before the incorporation of the reduced nitrogen to carbon skeletons. Induction of the entrance and reduction steps requires two nutritional conditions: the absence of ammonia, which is a metabolic repressor, and the presence of nitrate (nitrite), which acts as an inducer [1].

Both nitrate reductase and nitrite reductase genes from *A. vinelandii* [3], *Klebsiella pneumoniae* [4], and the cyanobacterium *Synechococcus* [5] have been cloned and shown to be included in the same operon. Furthermore, in *Synechococcus* [5,6] and, by analogy with it, in *K. pneumoniae* [4], a set of genes encoding the proteins involved in nitrate transport are organized in the operon of nitrate reductase and nitrite reductase genes. Very recently, two genes, *nasS* and *nasT*, involved in the expression of the nitrate and nitrite reductase operon (*nasAB*) of *A. vinelandii* have been identified, cloned and sequenced [7]. While the deduced NASS polypeptide is homologous to NRTA and to NASF, proteins involved in nitrate uptake in *Synechococcus* [6] and *K. pneumoniae* [4], respectively, the predicted NAST polypeptide is similar to the regulator proteins of the two component regulatory systems [8]. It was reported moreover that the expression of the *nasST* operon is not under the control of the NTR system and is not regulated by the nitrogen source.

In this work, we report the presence in *A. chroococcum* of two polypeptides with molecular masses of 22 and 35 kDa, respectively, which we term P22 and P35, the expression of which is regulated by the nitrogen source. P22 is associated with the cytoplasmic membrane and phosphorylated in response to nitrate, whereas P35 is a soluble component native to the periplasm. The results suggest that the P22 polypeptide might be a sensor protein involved in nitrate assimilation in *A. chroococcum*.

2. Materials and methods

$H_3{}^{32}PO_4$ (8800 Ci/mmol) and [^{35}S]methionine (1200 Ci/mmol) were purchased from Du Pont-New England Nuclear. Electrophoresis reagents and molecular weight markers were from Bio-Rad. All other chemicals were of analytical grade.

2.1. Organisms and growth conditions

A. chroococcum ATCC 4412 (from the Valencia University Collection, Valencia, Spain), *A. chroococcum* TR1 [9] and *A. chroococcum* MCD1 [10] were grown on nitrogen-free Burk's medium supplemented with 0.5% (w/v) sucrose as the sole energy and carbon source. When indicated, this medium was supplemented with NH_4Cl or KNO_3 at the concentration specified in each case. Growth conditions were as previously described [11].

2.2. Isolation of ^{35}S-labelled periplasmic fractions

Periplasmic fractions were prepared by two methods. To prepare fractions by the so-called 'mild treatment' [12] that, as we have shown previously, does not affect cell viability [1], *A. chroococcum* cells were grown for 2–4 h, as specified in each case, in media containing the appropriate nitrogen source in the presence of 5 μCi/ml [^{35}S]methionine. Cell suspensions were then harvested by centrifugation at 12 000 × *g* for 10 min (all centrifugations were at 4°C), washed with 10 mM Tris-HCl buffer, pH 8.0, and resuspended (1 g wet weight per 80 ml) in 20% (w/v) sucrose containing 30 mM Tris-HCl buffer, pH 8.0. Following 10 min of gentle agitation (90 rpm in a gyratory shaker) at 30°C, the cells were pelleted by centrifugation at 12 000 × *g* and the supernatant fluid was removed. The well-drained pellet was rapidly mixed with a volume of 15% (w/v) sucrose containing 30 mM Tris-HCl buffer, pH 7.5, equal to that of the original volume of the suspension and incubated for 5 min at 30°C. The mixture was centrifuged as above and the supernatant constituted the periplasmic fraction obtained by a 'mild treatment'. Purity of this fraction was checked by measuring the activities of glutamine synthetase and nitrite reductase, both soluble enzymes. None of them contaminated the periplasmic fractions. In addition, the presence of contaminating cytoplasmic membrane was discarded by the absence of any respiratory activity (NADH-dependent O_2 consumption). In agreement with previous data [1], these osmotically shocked *A. chroococcum* cells lost active transport processes (such as nitrate uptake and the active com-

*Corresponding author. Fax: (34) (5) 4620154.

PII S0014-5793(96)00843-5

ponent of nitrite uptake), but retained passive processes (such as ammonium uptake and the passive influx of nitrite).

Periplasmic fractions from the 'hard treatment', a treatment that decreases cell viability in *A. chroococcum*, were obtained essentially following the initial steps of the method previously described to prepare ^{35}S-labelled cytoplasmic membranes [1]. A 5 ml aliquot of the appropriate cell suspension was centrifuged at 12000×*g* for 10 min and the pellet washed twice with ice-cold 10 mM Tris-HCl buffer, pH 8.0. Cells were resuspended (1 g wet weight per 80 ml) at room temperature in 20% (w/v) sucrose containing 30 mM Tris-HCl, pH 8.0, 10 mM potassium EDTA, pH 7.0, 1 mg/ml lysozyme. This mixture was incubated at 30°C and the formation of spheroplasts monitored under a light microscope. After 30 min incubation more than 90% of the cells were spheroplasts. These were collected by centrifugation at 12000×*g* for 40 min, and the supernatant saved as the periplasmic fraction from the hard treatment. The purity of these fractions was checked as above referred to.

2.3. Isolation of cytoplasmic membrane fractions

After separating the periplasmic fraction from the hard treatment just described, the spheroplasts collected were used as the starting material to isolate the cytoplasmic membrane fraction. The experimental procedure has been described elsewhere [1], and the purity of the membrane fractions was checked by the absence of contaminating soluble enzymes such as glutamine synthetase and nitrate reductase. However, these fractions showed a high respiratory capacity.

To analyze periplasmic proteins, a 0.5 ml aliquot of the corresponding preparation was precipitated with 10% (w/v) trichloroacetic acid for 10 min at 0°C. The precipitate was pelleted at 12000×*g* for 15 min, and successively washed with absolute ethanol, ethanol/ether (1:1) and ethanol/ether (1:3), and then dissolved in sample buffer for electrophoresis [13]. Every fraction analyzed, i.e. periplasmic space, cytoplasmic membrane and soluble fraction, showed a characteristic pattern of polypeptides in SDS-PAGE analysis. This pattern served, in addition, to check the purity of these fractions.

2.4. In vivo ^{32}P-labelling experiments

These experiments were carried out as previously described [14] except that (a) after the addition of [^{32}P]orthophosphoric acid, and the subsequent incubation, the specified nitrogen source at the concentration also indicated was added, and (b) cells were treated with lysozyme to obtain the ^{32}P-labelled periplasmic fraction from hard treatment, as just described for the ^{35}S-labelling experiments. SDS-PAGE on 12% gels was performed according to Laemmli [13], proteins markers were electrophoresed in parallel, and bidimensional PAGE [15] and fluorograms of in vivo-labelled polypeptides [16] were obtained as already described. Where indicated the intensity of the P35 and P22 polypeptide autoradiogram bands was quantified using a computerized Bio Image system with the program 'Whole band analyzer'. 100% represents the value of the integrated intensity of each polypeptide in periplasmic fractions from N_2-fixing cells obtained by hard treatment. Protein was estimated by the method of Markwell et al. [17] using BSA as standard. All the results are representative of at least three separate experiments on different batches of bacteria.

3. Results and discussion

3.1. Two polypeptides, one of them located in the periplasm and the other one bound to the cytoplasmic membrane, the expression of which is dependent on the nitrogen source available to A. chroococcum

The fact that *A. chroococcum* TR1 strain, a mutant lacking one of the components of the nitrate transport system [1], exhibited activities of nitrate reductase and nitrite reductase when incubated with 8 mM KNO_3 [9] suggested that nitrate acted as an external stimulus. However, we had reported that nitrate induction in TR1 cells required 10-times higher nitrate concentration and longer incubation time than in wild-type cells [9]. A possibility was that this induction was due to the existence of a second, low affinity, transport system for nitrate. This possibility was discarded since TR1 cells were unable to grow at the expense of nitrate under an argon/oxygen atmosphere [9]. Therefore, nitrate must interact somehow with its own transport system to produce the induction of the nitrate assimilation pathway.

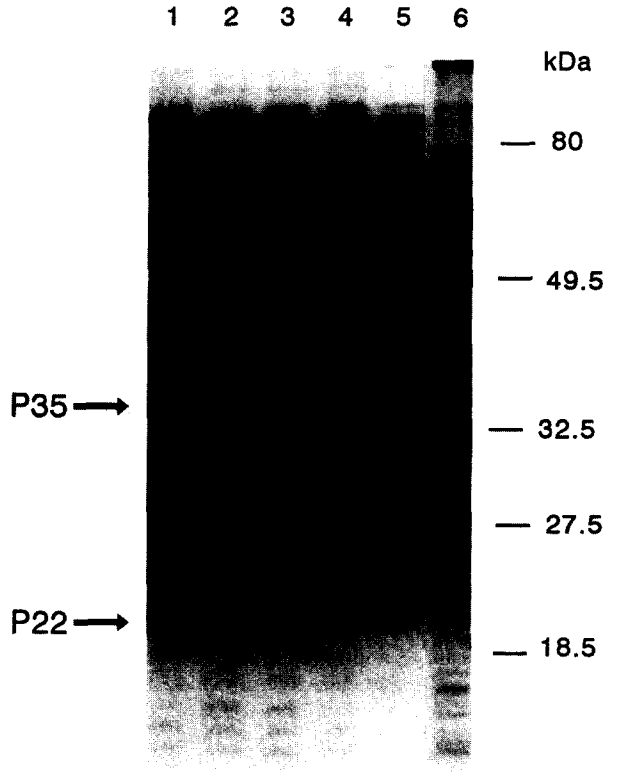

Fig. 1. Polypeptide composition of ^{35}S-labelled periplasmic fractions and cytoplasmic membranes upon transfer of ammonium-grown cells of *A. chroococcum* wild-type and TR1 mutant strain to media containing different nitrogen sources. Periplasmic fractions and cytoplasmic membranes were prepared from ammonium-grown cells that had been collected at an absorbance (A_{560}) of approx. 0.8, diluted with culture medium and then subcultured for 4 h (A_{560} 0.3) in a medium containing 5 μCi/ml [^{35}S]methionine and the indicated nitrogen source. Lanes 1–3, periplasmic proteins ('hard treatment') from wild-type cells transferred to combined nitrogen-free medium (lane 1), 10 mM KNO_3 (lane 2), 10 mM NH_4Cl (lane 3). Lane 4, periplasmic proteins (hard treatment) from strain TR1 transferred to nitrate (10 mM) medium. Lane 5, periplasmic proteins from wild-type cells transferred to combined nitrogen-free medium and subjected to an osmotic shock in the absence of lysozyme ('mild treatment'). Lane 6, cytoplasmic membrane proteins from *A. chroococcum* wild-type cells transferred to combined nitrogen-free medium (see Section 2 for details).

This reasoning prompted us to investigate what role some component present in either the periplasm or the cytoplasmic membrane may play in the nitrate induction of nitrate assimilation. For this purpose, we first analyzed by SDS-PAGE and autoradiography the polypeptide composition of periplasmic preparations from *A. chroococcum* cells that had been grown with ammonia as nitrogen source and afterwards incubated for 4 h with either ammonia, nitrate, or just under air, in the presence of [^{35}S]methionine. To isolate the periplasmic fraction, cells were then collected and osmotically shocked in the presence of lysozyme, as above referred to. Two polypeptides with molecular masses of 22 and 35 kDa, that we termed P22 and P35, respectively, were very abundant in periplasmic preparations from cells that had been incubated under N_2-

fixing conditions (Fig. 1, lane 1) and at very low levels in preparations from cells incubated with nitrate (lane 2) or ammonia (lane 3). Interestingly, they were present at high level in preparations from the TR1 mutant incubated with nitrate (lane 4) showing that nitrate utilization, which is not possible in TR1 cells, was necessary to exert the repressor effect on both polypeptides observed in the wild-type cells. Therefore, the expression of P22 and P35 shows a regulation by the nitrogen source similar to that of nitrogenase activity: they are abundant under N_2-fixing conditions and scarce in medium supplemented with nitrate or ammonia.

In periplasmic preparations obtained by osmotic shock in the absence of lysozyme, P35 was as abundant as in fractions prepared in its presence (Fig. 1, lane 5). However, P22 was almost undetectable (see also Fig. 1, lane 5). Analysis of the polypeptide content of the cytoplasmic membrane preparations from cells incubated in combined nitrogen-free medium revealed that a significant amount of P22 was in this fraction while P35 was very scarce (lane 6). These results allow us to conclude that one of the two polypeptides whose expression is regulated by the nitrogen source, P35, is soluble while the other, P22, is associated with the cytoplasmic membrane, although it is not an integral membrane-bound protein.

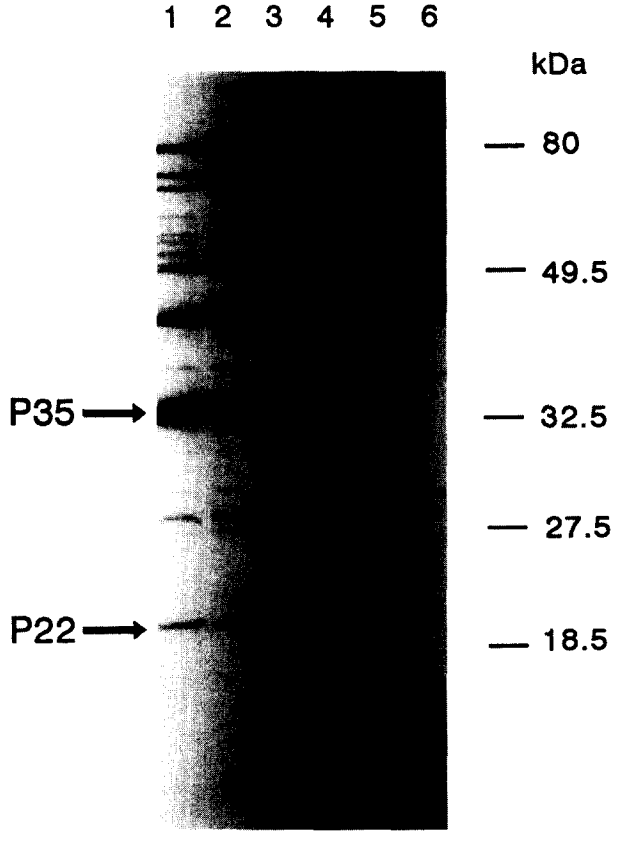

Fig. 2. Effect of the nitrogen source and the incubation time on de novo synthesis of P22 and P35 in *A. chroococcum* ATCC 4412. Lanes 1 and 2 contained periplasmic fraction ('hard treatment') proteins from wild-type cells grown under N_2-fixing conditions (A_{560} of approx. 0.8), then transferred to combined nitrogen-free medium (lane 1) or 10 mM KNO_3 (lane 2), each one containing 5 μCi/ml [^{35}S]methionine, and incubated under growth conditions for 1 h. Lanes 3 and 4 as in lanes 1 and 2, respectively, but incubated for 2 h. Lanes 5 and 6 contained periplasmic proteins ('hard treatment') from ammonium-grown cells, transferred to combined nitrogen-free medium (lane 5) or 10 mM KNO_3 (lane 6) and cultured for 1 h. Experimental conditions as in Fig. 1 and Section 2.

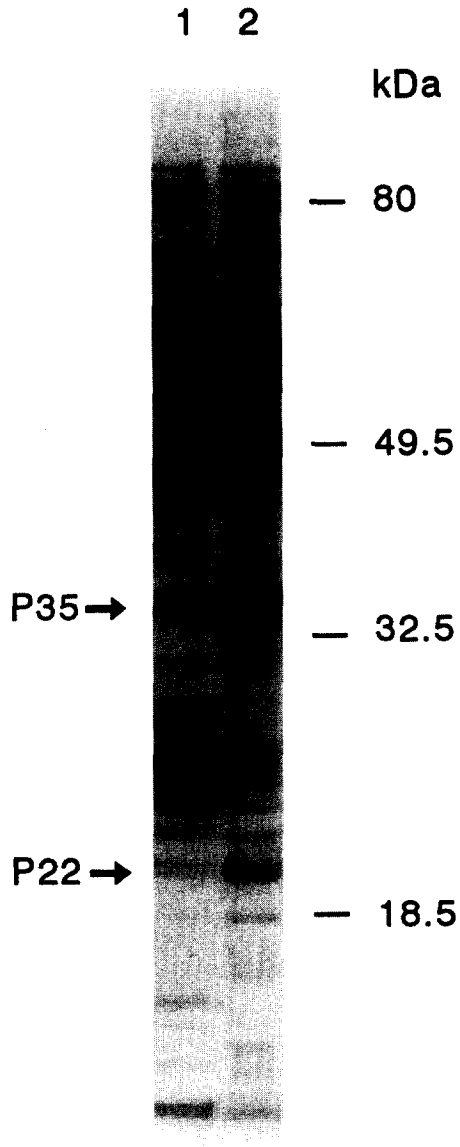

Fig. 3. Polypeptide composition of ^{35}S-labelled periplasmic fractions from cells of *A. chroococcum* mutant strains MCD1 and TR1. Periplasmic fractions ('hard treatment') were prepared from cells that had been harvested at an absorbance (A_{560}) of approx. 0.8, diluted with culture medium and then subcultured for 2 h in a medium containing 5 μCi/ml [^{35}S]methionine and 10 mM KNO_3. Lane 1, periplasmic proteins from MCD1 cells. Lane 2, periplasmic proteins from TR1 cells.

3.2. *Regulation of P22 and P35 synthesis by the nitrogen status of the cells*

It was expected that if wild-type cells grown under N_2-fixing conditions, and hence exhibiting high levels of both P22 and P35 polypeptides, were transferred to nitrate medium, the synthesis of these proteins would not decrease until the nitrate assimilation system reached a significant induction level to metabolize the anion. That this was the case is shown in Fig. 2, which demonstrates that after 1 h incubation in the presence of 10 mM KNO_3 (lane 2) the intensity of both polypeptides, in comparison with that displayed under N_2-fixing conditions (lane 1), decreased scarcely, as measured by densi-

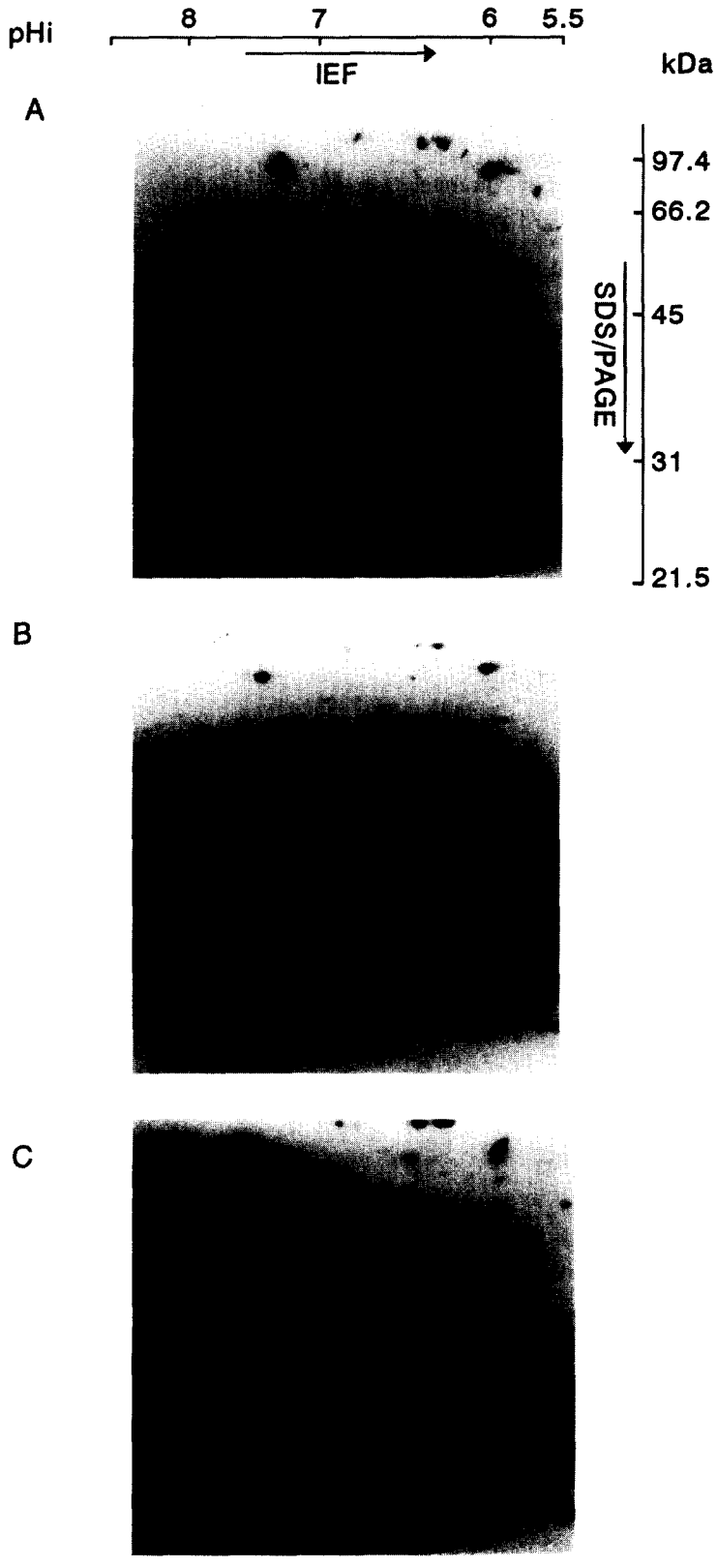

Fig. 4. Bidimensional electrophoresis analysis of P22 and P35 in periplasmic preparations from cells of *A. chroococcum* wild-type and TR1 mutant strain. Periplasmic preparations ('hard treatment') were obtained from N_2-fixing cells of *A. chroococcum* wild-type that had been collected (A_{560} approx. 0.8), then transferred to a medium supplemented with 5 μCi/ml [^{35}S]methionine and afterwards cultured for 2 h in the presence of the following nitrogen source: air-nitrogen (A), 10 mM KNO_3 (B). Cells of the TR1 strain were subjected to the same treatment and transferred to a medium containing 10 mM KNO_3 (C). Arrows on the autoradiograms denote the polypeptides of 22 and 35 kDa, and three reference proteins, R1–R3. The pH range of the isoelectrofocusing running (IEF) and protein markers for the SDS-PAGE are shown on the top and right margins, respectively.

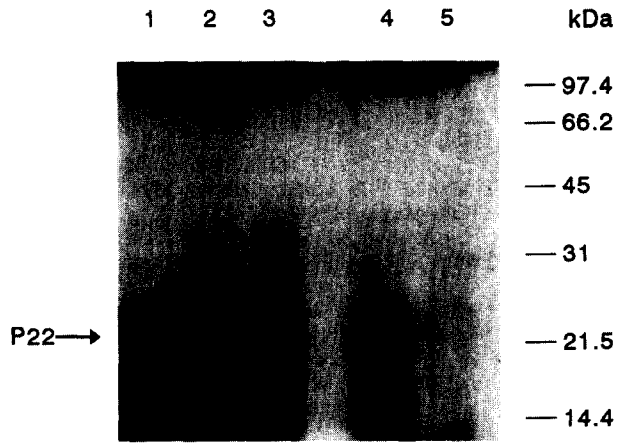

Fig. 5. Nitrate-dependent ^{32}P labelling of P22 in cells of wild-type and TR1 strains. Lanes 1–5 contained periplasmic proteins ('hard treatment') from N_2-fixing *A. chroococcum* wild-type cells that had been collected at an A_{560} of approx. 0.8 and then incubated under growth conditions during 1 h in the presence of either 0.5 mM KNO_3 (lane 1), 1 mM $NaNO_2$ (lane 2), 1 mM NH_4Cl (lane 3), 1 mM urea (lane 5) or in a combined nitrogen-free medium (lane 4) (see Section 2 for details).

tometry scanning of the bands, while after 2 h incubation with nitrate P35 diminished by approx. 40% and P22 by 30% (lanes 3,4 as lanes 1,2, respectively, but during 2 h incubation). As a control, cells arising from a medium supplemented with ammonia, and therefore without expressing P22 and P35 (see above), exhibited synthesis of both polypeptides after 1 h incubation under N_2-fixing conditions (lane 5) but not in the presence of KNO_3 (lane 6). These results provide strong support that the synthesis of both polypeptides is regulated by the cellular carbon/nitrogen balance, the greatest expression being reached when cells are in the presence of a nitrogen source, such as molecular nitrogen, whose utilization is a very energy-demanding biological process.

To corroborate further the involvement of P22 and P35 in nitrate assimilation, *A. chroococcum* MCD1 [10] was used. This strain is unable to use nitrate or nitrite as the sole nitrogen source under an Ar/O_2 atmosphere. When grown under air in the presence of nitrate, MCD1 cells exhibited undetectable nitrate (nitrite) uptake activity and negligible levels of nitrate reductase and nitrite reductase activities, whereas nitrogenase activity was similar to that found in wild-type *A. chroococcum* ATCC 4412 [18]. It is not yet known what kind of mutation produces this phenotype in MCD1 cells, but a possibility is that this strain lacks any component required for the induction of the nitrate assimilation system. Therefore, the presence of P22 and P35 was investigated in periplasmic fractions from this mutant and the results compared with those of the TR1 strain. Fig. 3 (lane 1) shows that P22 was missing from, and P35 very much decreased in, MCD1 periplasmic preparations. Under the same experimental conditions, periplasmic preparations from *A. chroococcum* TR1 (Fig. 3, lane 2) showed high levels of both proteins, as also illustrated in Fig. 1 (lane 4). These results lend support to the proposal that P22 and P35 may be required for induction of the nitrate assimilation pathway.

3.3. P22 is phosphorylated in response to nitrate (nitrite)

Periplasmic preparations from cells of *A. chroococcum* wild-type and TR1 mutant strains grown under N_2-fixing conditions, then incubated with [^{35}S]methionine in the presence of different nitrogen sources for 2 h and afterwards collected and treated with lysozyme were analyzed by bidimensional PAGE. In comparison with its location on the autoradiogram from N_2-fixing cells (Fig. 4A), a shift of P22 to the more acidic part of the gel was observed after transferring the bacteria to nitrate medium (Fig. 4B), thus suggesting that it was covalently modified by a small, negatively charged group. No change in the position of P22 was apparent in preparations from cells that had been transferred again to N_2-fixing conditions (Fig. 4A). The change in mobility is clearly observed comparing the position of P22 with that of the protein denoted R1 in Fig. 4A, B and (see below) C. In the absence of nitrate, P22 and the reference protein R1 appeared aligned. In cells transferred to nitrate medium, P22 moved to the acidic zone. Other alignment controls, such as that of R2 and R3 proteins, were conserved independently of the nitrogen source in the medium, as shown in Fig. 4A and B. Interestingly, the shift of P22 was observed in periplasmic preparations from TR1 mutant cells (Fig. 4C) grown at the expense of air-nitrogen in the presence of nitrate.

Fig. 4 also illustrates that nitrate did not promote any change in the electrophoretic mobility of P35 when N_2-fixing wild-type *A. chroococcum* cells (Fig. 4A) were transferred to nitrate medium (Fig. 4B). Furthermore, P35 from the *A. chroococcum* TR1 mutant (Fig. 4C) was found to be aligned with that from the wild-type strain (Fig. 4A,B).

The results just described strongly suggest that P22 could act in *A. chroococcum* as a sensor for the presence of the inducer nitrate. In the two-component regulatory systems, the sensor modification promoted by the stimulus is a phosphorylation of a specific amino acid residue. We examined therefore whether the above-described P22 modification in response to nitrate consisted of phosphorylation. Fig. 5 shows that P22 was in vivo labelled in *A. chroococcum* cells that had been grown diazotrophically and then incubated with either 0.5 mM KNO_3 (lane 1) or 1 mM $NaNO_2$ (lane 2) in the presence of [^{32}P]orthophosphate during 1 h, but not in the presence of 1 mM NH_4Cl (lane 3) or 1 mM urea (lane 5). The phosphorylation of P22 was clearly detected at 15 min from the addition of nitrate (nitrite) to the cells, thus distinguishing this type of rapid response from long-term metabolic effects. In the absence of combined nitrogen, labelling of P22 (lane 4) was approx. 10% of that achieved with $NaNO_2$ and 40% with respect to KNO_3, as measured by densitometry scanning of the bands. No ^{32}P labelling of any polypeptide with a molecular mass within 30–40 kDa was observed. These data therefore show that nitrate (nitrite) promoted the in vivo phosphorylation of P22.

From the present results, it follows that even though nitrate does not have to be metabolized, it must interact in some way with the nitrate transport system [1] to result in the covalent modification of P22 and the subsequent expression of the nitrate assimilation system, as proposed above. In connection with this point, it has been reported that in vitro phosphorylation of NarX, a sensor for nitrate in *E. coli*, is not influenced by nitrate, though NarX activity is regulated by nitrate in vivo [19]. To explain this finding, it was suggested that nitrate regulation of NarX phosphorylation will most likely require reconstitution of a nitrate-dependent form of NarX in the cell membrane [20].

In the two-component systems, the signal monitored by the

sensor is transmitted to a regulatory component. In the case of *A. chroococcum* nitrate assimilation, a possibility is that NtrC acts as a regulatory component. This possibility is favoured by the fact that NtrC is required for the transcription of the genes encoding nitrate and nitrite reductase in *A. vinelandii* [21,22]. The fact that the *A. vinelandii nasST* operon is not regulated by the nitrogen source, as mentioned above, stands against the possibility that the NAST polypeptide, the molecular mass of which is 21.6 kDa [7], has something else in common with P22. Work is now in progress to understand the nitrate transduction signal and its relationship with the NTR system and to determine what role, if any, P35 may play in the regulation of expression of nitrate assimilation.

Acknowledgements: This work was aided by grant BIO91-0914 from Comisión Interministerial de Ciencia y Tecnología and by financial aid from Plan Andaluz de Investigación (Spain). The secretarial assistance of Pepa Pérez de León and Reyes Domínguez is deeply appreciated.

References

[1] Muñoz-Centeno, M.C., Cejudo, F.J., Ruiz, M.T. and Paneque, A. (1993) Biochim. Biophys. Acta 162, 356–368.
[2] Guerrero, M.G., Vega, J.M. and Losada, M. (1981) Annu. Rev. Plant Physiol. 32, 169–204.
[3] Ramos, F., Blanco, G., Gutierrez, J.C., Luque, F. and Tortolero, M. (1993) Mol. Microbiol. 8, 1145–1153.
[4] Lin, J.T., Goldman, B.S. and Stewart, V. (1994) J. Bacteriol. 176, 2551–2559.
[5] Luque, I., Herrero, A., Flores, E. and Madueño, F. (1992) Mol. Gen. Genet 232, 7–11.
[6] Omata, T., Andriesse, X. and Hirano, A. (1993) Mol. Gen. Genet. 236, 193–202.
[7] Gutiérrez, J.C., Ramos, F., Ortner, L. and Tortolero, M. (1995) Mol. Microbiol. 18, 579–591.
[8] Parkinson, J.S. (1993) Cell 73, 857–871.
[9] Ruiz, M.T., Cejudo, F.J., Muñoz-Centeno, M.C. and Paneque, A. (1990) FEMS Microbiol. Lett. 67, 211–214.
[10] Robson, R.L., Chesshyre, J.A., Wheeler, C., Jones, R., Woodley, P.R. and Postgate, J.R. (1984) J. Gen. Microbiol. 130, 1603–1612.
[11] Paneque, A., Bárcena, J.A., Cordero, N., Revilla, E. and Llobell, A. (1982) Mol. Cell. Biochem. 49, 33–41.
[12] Kaback, H.R. (1971) Methods Enzymol. 22, 99–120.
[13] Laemmli, U.K. (1970) Nature 227, 680–685.
[14] Muñoz-Centeno, M.C., Cejudo, F.J. and Paneque, A. (1994) Biochem. J. 298, 641–645.
[15] O'Farrel, P.H. (1975) J. Biol. Chem. 250, 4007–4021.
[16] Bonner, W.M. and Laskey, R.A. (1974) Eur. J. Biochem. 46, 83–88.
[17] Markwell, M.A.K., Hass, S.M., Bieber, L.L. and Tolbert, N.E. (1978) Anal. Biochem. 87, 206–210.
[18] Cejudo, F.J. and Paneque, A. (1986) J. Bacteriol. 165, 240–243.
[19] Parkinson, J.S. and Kofoid, E.C. (1992) Annu. Rev. Genet. 26, 71–112.
[20] Walker, M.S. and DeMoss, J.A. (1993) J. Biol. Chem. 268, 8381–8383.
[21] Toukdarian A. and Kennedy, C. (1986) EMBO J. 5, 399–407.
[22] Luque, F., Santero, E., Medina, J.R. and Tortolero, M. (1987) Arch. Microbiol. 148, 231–235.