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Posttranslational regulation of nitrogenase activity by fixed nitrogen in *Azotobacter chroococcum*

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Abstract

Using anti-(Fe protein) antibody raised against the Fe protein of the photosynthetic bacterium *Rhodospirillum rubrum*, it was found that the Fe protein component of nitrogenase (EC 1.18.2.1) from *Azotobacter chroococcum* cells subjected to an ammonium shock, and hence with an inactive nitrogenase, appeared as a doublet in Western blot analysis of cell extracts. The Fe protein incorporated [^{32}P]phosphate and [^{3}H]adenine in response to ammonium treatment, and L-methionine-DL-sulfoximine, an inhibitor of glutamine synthetase (L-glutamate: ammonia ligase (ADP forming), EC 6.3.1.2), prevented Fe protein from inhibition and radioisotope labelling. These results support that *A. chroococcum* Fe protein is most likely ADP-ribosylated in response to ammonium. After ammonium treatment, when in vivo activity was completely inhibited, Fe-protein modification was still increasing. This suggests the existence of another mechanism of nitrogenase inhibition faster than Fe-protein modification. When ammonium was intracellularly generated instead of being externally added, as occurs with the short-term nitrate inhibition of nitrogenase activity observed in *A. chroococcum* cells simultaneously fixing molecular nitrogen and assimilating nitrate, a covalent modification of the Fe protein was likewise demonstrated.

Keywords: Ammonium; Nitrate; Nitrogen fixation; Nitrogenase; (*A. chroococcum*)

1. Introduction

Atmospheric nitrogen is reduced to ammonia by the nitrogenase enzyme complex in a small number of procaryotic microorganisms. Since the biological nitrogen fixation is a very energy-demanding process, it is not surprising that nitrogenase is regulated tightly, both at the transcriptional [1] and posttranslational level [2].

One of the nitrogenase activity control systems that is being thoroughly studied is its reversible inhibition by ammonium, that was first described some 50 years ago [3]. Thus addition of a little amount of ammonium to a culture of an organism reducing molecular nitrogen results in a rapid inhibition of nitrogen fixation. Once the added ammonium is exhausted from the medium nitrogenase resumes its catalytic activity. Though this phenomenon was discovered in *Azotobacter vinelandii* [3], it was erroneously believed for some time to be peculiar to photosynthetic bacteria, but now is recognized to occur in free-living diazotrophs such azotobacters [4,5], azospirilla [6], cyanobacteria [7], and symbiotic bacteria in ex-planta cultures [8], and it has been termed 'switch on/off' ([9,10] and references therein)

The molecular mechanism of nitrogenase 'switch on/off' has been extensively investigated, among others, in the phototroph *Rhodospirillum rubrum*, in which it involves reversible mono-ADP-ribosylation of the Fe protein component of nitrogenase in response to the addition of ammonium [2]. Briefly, the ADP-ribose moiety of NAD^+ is transferred by an (Fe protein)-ADP-ribosyltransferase to the Arg-101 residue of one subunit of the Fe protein dimer of *R. rubrum* and thus inactivates the nitrogenase enzyme complex. Removal of the ADP-ribose from the covalently modified nitrogenase component by an

Abbreviations: GS, glutamine synthetase; GOGAT, glutamate synthase; Hepes, (*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]); Mops, 3-morpholinepropane sulfonic acid; MTAB, mixed alkyltrimethylammonium bromides; MSX, L-methionine-DL-sulfoximine

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(Fe protein)-activating glycohydrolase restores its activity. This modification/demodification can be easily followed in photosynthetic bacteria: SDS/PAGE analysis of the Fe protein from NH_4^+-limited cells and hence with an active nitrogenase enzyme shows the presence of a single type of subunit, whereas the Fe protein from NH_4^+-shocked cells appears as two polypeptide chains with differential migration on SDS/PAGE [2].

The short-term ammonium inhibition of nitrogenase activity in some organisms, such as *Azopirillum amazonense* and *Herbaspirillum seropedicae* [10], is due to indirect effects on the nitrogenase reaction and does not involve covalent modification of the Fe protein. Recently evidence has been presented on the existence of the two responses of nitrogenase to ammonium in the same bacterium, *Rhodobacter capsulatus* [11].

It has been reported that, in *A. vinelandii*, the two subunits of the Fe protein component appeared as two polypeptide chains with slightly different mobility on SDS/PAGE stained gels. However, no relation between the presence or absence of these two bands and the activity of the Fe protein was found [12]. In fact it has been emphasized that there is no evidence for in vivo covalent modification of the *A. vinelandii* Fe protein [10]. Nevertheless, using the (Fe-protein)-ADP-ribosyltransferase from *R. rubrum*, a covalent modification of *A. vinelandii* Fe protein was shown to occur in vitro [13].

In this report, we present data strongly supporting that, in *A. chroococcum*, ammonium inhibition of nitrogenase activity is mediated, at least, by an ADP-ribosylation of the Fe protein, that becomes separated into two closely migrating bands in immunoblot analysis. We present also results in favour that the short-term nitrate inhibition of *A. chroococcum* nitrogenase activity, previously described by this group [14], is due to a covalent modification of Fe protein, most probably an ADP-ribosylation induced by the ammonium generated in nitrate assimilation. In situ assays of nitrogenase activity inhibited by ammonium in vivo revealed that the enzyme was mostly present in an inactive, covalently modified, form.

2. Methods

2.1. Materials

ADP, ATP, Mops, MTAB, Hepes, Tris, and Tricine were purchased from Sigma. [^{3}H]Adenine (25.8 Ci/mol) and $H_3^{32}PO_4$ (8800 Ci/mmol) were from New England Nuclear. Electrophoresis reagents and molecular weight markers were from BioRad. All other chemicals were of analytical grade from Merck.

2.2. Organisms and culture conditions

A. chroococcum ATCC 4412 (from the Valencia University Collection, Valencia, Spain) was used in this study. Cells were grown heterotrophically on nitrogen-free Burk's medium supplemented with 0.5% (w/v) sucrose as the sole energy and carbon source. When necessary this medium was supplemented with either potassium nitrate or ammonium chloride at the concentration indicated in each case. Growth conditions were as previously described [15]. Mid-logarithmic-growth cells were used for each experiment.

2.3. Cell extracts

Cells were harvested by centrifugation at $10\,000 \times g$ for 10 min at 4°C, washed in 50 mM Mops-KOH buffer, pH 7.5, and resuspended at a concentration of 1 g of cells per 3 ml of 50 mM Mops-KOH buffer, pH 7.5, containing 1 mM DL-dithiothreitol and 1 mM phenylmethanesulfonyl fluoride. Bacteria were then disrupted by freezing in liquid air followed by boiling for 3 min (repeated four times at least). The homogenate was centrifuged at $33\,000 \times g$ for 10 min at 4°C and the supernatant constituted the cell extract.

2.4. Western blot (immunoblotting)

After SDS/PAGE, the proteins from samples of cell extracts were transferred to nitrocellulose sheets as in [16], using a mini-Trans Blot electrophoretic transfer cell (Bio-Rad). To immunodetect proteins, the nitrocellulose filters were blocked overnight in 200 mM NaCl and 15 mM Tris-HCl, pH 7.4 (Tris-NaCl), containing 0.2% (w/v) sodium azide and 5% (w/v) dried skimmed milk. Anti-(Fe protein) antibody (1:500) directed against the *R. rubrum* Fe protein (kindly provided by Dr. P.W. Ludden through Dr. J. Imperial) was added, and the mixture incubated with shaking overnight. The filters were washed four times with Tris-NaCl containing 0.05% (v/v) Tween-20, then peroxidase-conjugated anti-(rabbit IgC) serum (Sigma) was used as second antibody and blots were developed as described by Kombrink et al. [17].

2.5. In vivo $^{32}P_i$-labelling ([^{3}H]adenine-labelling) experiments

A 2 ml culture was grown to mid-logarithmic phase (A_{560} of approx. 0.5) in nitrogen-free medium. Cells were harvested by centrifugation at $7000 \times g$ for 5 min at room temperature and resuspended in 1 ml of culture medium buffered with 50 mM Mops-KOH, pH 7.5, and without any potassium phosphate. Cells were incubated at 30°C with continuous shaking (100 strokes min^{-1}) for 10 min and then supplemented with either 2 μCi of [^{32}P]orthophosphoric acid or 19 μCi of [^{3}H]adenine. After further incubation for 2 h, 5 mM NH_4Cl was added and the cells were harvested 1 h later. Sedimentation was improved by adding NaCl and EDTA to final concentrations of 100 mM and 10 mM, respectively. The cells were resuspended in 40 μl of sample buffer [18] for electrophoresis and dis-

rupted as above. The homogenate was centrifuged at 16500 × *g* for 20 min and the supernatant constituted the labelled cell extract.

2.6. Nitrogenase assay

Nitrogenase activity measurements were carried out using two cell suspensions of 2.5 ml each one at a concentration of approx. 1 mg cell protein per ml of 50 mM Hepes-KOH buffer, pH 7.5, containing 1% (w/v) sucrose. For the in vivo nitrogenase activity assay the two cell suspensions were preincubated for 15 min in 10 ml sealed conical flasks at 27°C with continuous shaking (100 strokes min^{-1}). The reaction was started by making the air gas phase 10% in acetylene and ethylene production followed by gas chromatography [19]. At the time indicated in each experiment, 5 mM either NH_4Cl or KNO_3 was added to one of the cell suspensions to inhibit nitrogenase activity and the in vivo measurements were followed during the time opportunely specified. The cell density used in this study (1 mg per ml) is suboptimal for nitrogenase assay, nevertheless it was chosen because it allowed to carry out Fe protein labelling experiments, that required such high cell density, in parallel with in vivo assay of nitrogenase activity. For the in situ nitrogenase activity assay 0.5 mM DTT and 12 mM $Na_2S_2O_4$ (final concentrations) were then added to both cell suspensions and two 0.6 ml aliquots of these preparations were injected respectively into two flasks fitted with rubber stoppers (total capacity 10 ml) that had been previously flushed with argon to achieve complete anaerobiosis. 0.2 ml of a solution that contained in 1 ml: $Na_2S_2O_4$, 50 μmol; Hepes/KOH buffer, pH 7.5, 100 μmol; and MTAB, 100 μg, was then added to each flask and the suspensions shaken for a few seconds. After that, 0.2 ml of an ATP regenerating mixture was added to each flask and the resulting suspension incubated at 30°C with continuous shaking (100 strokes min^{-1}) during 5 min. The reaction was then started by the addition of 1.5 ml acetylene and the amount of ethylene produced was determined as above. The ATP-regenerating mixture was prepared just before using by the combination of the immediately described solutions I and II in the proportion of 9:1. The solution I contained in 1 ml: bovine serum albumin, 5 mg; creatine kinase, 0.25 mg and in μmol, Hepes/KOH buffer, pH 7.5, 50; $MgCl_2$, 50; creatine phosphate, 125; and NaOH, 12. Solution II contained, in 1 ml of 50 mM Hepes/KOH buffer, pH 7.5, ATP, 150 μmol. All the results are representative of at least three separate experiments on different batches of bacteria.

2.7. Analytical methods

Samples of cell extracts containing approx. 40–60 μg protein were subjected to SDS/PAGE on 10% gels. Electrophoresis was performed in gel slabs according to Laemmli [18]. Protein markers were electrophoresed in parallel. Fluorograms of in vivo labelled polypeptides were obtained as already described [20]. Where indicated the intensity of the Fe protein autoradiogram band was quantified using a computerized Bio Image system with the program 'Whole band analyzer'.

Protein was estimated by the method of Markwell et al. [21] using bovine serum albumin as standard. Ammonium consumption was determined by following the disappearance of the ion from the medium as previously described [5].

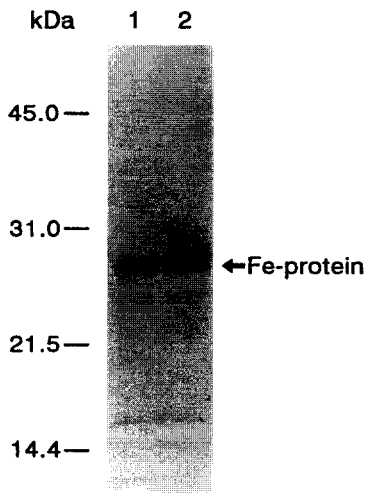

Fig. 1. Western blot-analysis of extracts from ammonium-treated *A. chroococcum* cells using anti-(Fe protein) antibodies. Diazotrophically grown *A. chroococcum* cells were treated with 5 mM NH_4Cl for 1 h to inactivate nitrogenase and then used to obtain the corresponding cell extract. Aliquots (59 μg of protein) of the extract from untreated cells (lane 1) and from ammonium-treated cells (lane 2) were electrophoresed in an SDS/10%-polyacrylamide gel, and the Western blot was carried out as described in Section 2. The molecular masses of protein standards are indicated on the left margin.

3. Results and discussion

3.1. Identification by immunologic techniques of the Fe protein component of nitrogenase in extracts from A. chroococcum cells subjected to an ammonium shock

Cell extracts from N_2-fixing *A. chroococcum* cells that had been supplemented with 5 mM NH_4Cl and then incubated during 60 min under growth conditions, and therefore without any nitrogenase activity [5], were electrophoresed on SDS/PAGE and subjected to Western blot using anti-(Fe protein) antibodies raised against the Fe protein from *R. rubrum*. Fig. 1 shows that the *A. chroococcum* Fe protein in extracts from cells treated with NH_4^+ (lane 2) appeared as a doublet closely resembling other Fe proteins when they are modified by ADP-ribosylation. In this doublet the polypeptide of higher molecular mass would correspond to the modified subunit. Al-

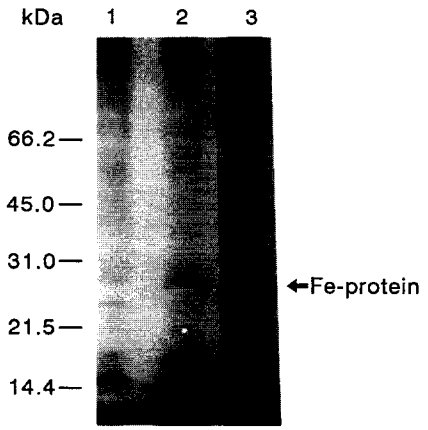

Fig. 2. Incorporation of [^{32}P] in the inactive Fe protein component of *A. chroococcum* ATCC 4412 and its prevention by MSX. $^{32}P_i$ labelling of diazotrophically-grown cells was carried out as described in Section 2. The cell extracts thus obtained (50 μg of protein) were electrophoresed in an SDS/10%-polyacrylamide gel and the gel subjected to autoradiography. Lanes: 1, cell extract of non-ammonium treated cells (containing active nitrogenase); 2, cell extract from cells treated with 5 mM NH_4Cl for 1 h (containing inactive nitrogenase); 3, cell extract from bacteria treated with 5 mM MSX 40 min prior to the NH_4Cl addition (containing active nitrogenase). The molecular masses of protein standards are indicated on the left margin.

though to a lesser extent, in extracts from control cells, non-treated with ammonium, some modified Fe protein was present (Fig. 1, lane 1). This pattern was also observed when cells were broken with methods, such as osmotic shock, less dramatic than repeated boiling, that is, a clear doublet in ammonium treated cells and some modified Fe-protein in control cells. Similar results have been described in the analysis of Fe protein modification in *Azospirillum brasilense* and *Azospirillum lipoferum* [6]. To explain the appearance of some modified Fe protein in non-ammonium treated cells, these authors discuss that it is due to partial inactivation of nitrogenase that takes place during cells harvest and breakage; most probably this is also the reason for the detection of some modified Fe protein in non-ammonium treated *A. chroococcum* cells. As shown in Fig. 1, the antiserum was equally effective in the recognition of both active and inactive Fe protein form.

3.2. *In vivo* $^{32}P_i$-labelling ([^{3}H]adenine-labelling) of the Fe protein component of A. chroococcum nitrogenase, and its prevention by MSX

When diazotrophically-grown *A. chroococcum* cells were preincubated in the presence of $H_3^{32}PO_4$ and then subjected to an ammonium shock, 5 mM NH_4Cl during 1 h, the polypeptide previously identified as Fe protein modified subunit was labelled (Fig. 2, lane 2). No significant radioactivity was detected in this region when the cells were not given the ammonium treatment (lane 1). These results indicated that the Fe protein was modified by a group containing phosphate. Furthermore, when cells were supplemented with [^{3}H]adenine, the Fe protein was radiolabelled in response to ammonium addition in a similar

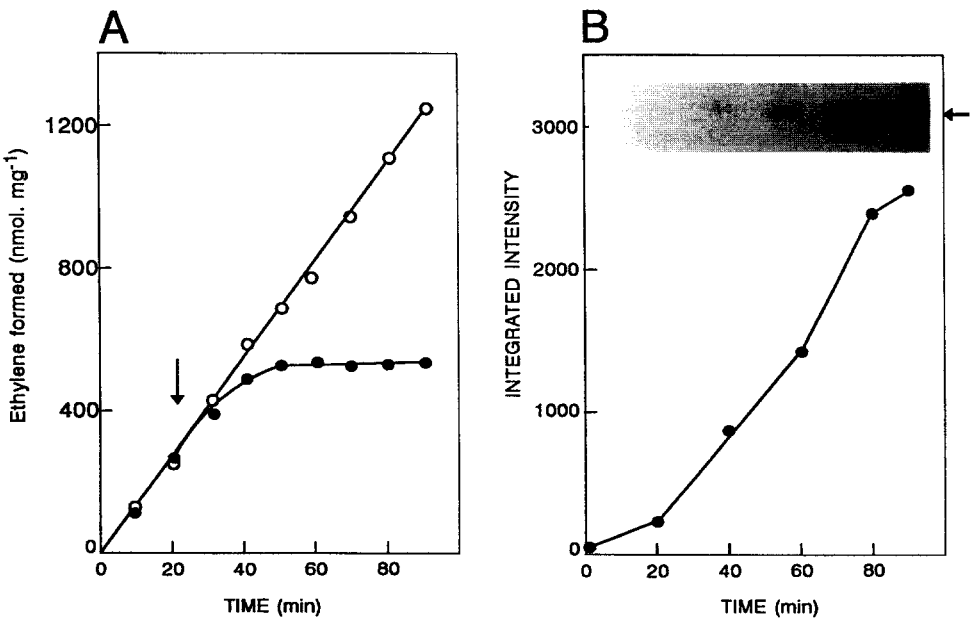

Fig. 3. Time course of in vivo ammonium inhibition of nitrogenase activity and of the concomitant $^{32}P_i$ labelling of Fe protein. Diazotrophically-grown *A. chroococcum* ATCC 4412 cells were used to parallely follow ammonium inhibition of nitrogenase activity (A) and $^{32}P_i$ labelling of Fe protein with its quantification (B). Nitrogenase activity was determined in the absence (○) and in the presence (●) of 5 mM NH_4Cl, which was added when indicated by the arrow (part A). $^{32}P_i$ labelling of the cells was carried out as described in Section 2. The cells extracts (50 μg of protein) were electrophoresed in an SDS/10%-polyacrylamide gel and the gel subjected to autoradiography (inset in part B). Arrow points to the Fe protein component band. The integrated intensity values of this band versus time of incubation of the cell in the presence of ammonium are given in part B.

way to that described in Fig. 2, indicating that the modifying group also contained adenine (not shown).

In *A. vinelandii* [22] and *A. chroococcum* [23], ammonium is incorporated into carbon skeletons via the GS-GOGAT (EC 1.4.7.1) route. In *A. chroococcum* cells that had been preincubated with MSX, an extensively used inhibitor of GS activity, ammonium was without effect on either nitrogenase activity [5] or nitrate uptake [24], the conclusion being drawn that ammonium must be metabolized to exert its inhibitory effect on these processes. It was expected therefore that incorporation of $H_3^{32}PO_4$ or radiolabelled adenine into Fe protein would be prevented by MSX. Lane 3 in Fig. 2 shows that $^{32}P_i$ labelling of Fe protein in cells subjected to an ammonium shock in the presence of MSX did not take place. The same results were obtained in experiments on [^{3}H]adenine-labelling of Fe protein during ammonium inactivation (not shown).

3.3. Comparative studies between the kinetics of in vivo inhibition of nitrogenase activity and of Fe protein radioactive-labelling

To go deeply into the relationship between ammonium inhibition of nitrogenase activity and covalent modification of the Fe protein component, a parallel study on the kinetics of both processes was undertaken. For such purpose a cell density of approx. 1 mg protein per ml was chosen so that it was posible to carry out parallel determinations of nitrogenase activity in vivo (Fig. 3A) and Fe protein labelling (Fig. 3B). Under these assay conditions the nitrogenase activity (13.3 nmol ethylene · mg^{-1} · min^{-1}) was significantly lower than activities previously described by our group for *A. chroococcum* [5] using optimal cell density of approx. 0.07 mg protein per ml. This is most probably due to oxygen limitation since it has been described that nitrogenase activity and its inhibition by ammonium in *A. vinelandii* is highly dependent on culture and assay conditions such as oxygen availability, respiration rate, or pH [25]. Under these assay conditions the addition of ammonium promoted nitrogenase activity inhibition, so that 100% inhibition was achieved 30 min after the addition of ammonium (Fig. 3A). Fig. 3B (inside) illustrates that $^{32}P_i$ labelling of Fe protein as a function of time was not so fast. To follow with precision the kinetics of [^{32}P]phosphate incorporation into the Fe protein the autoradiogram shown in Fig. 3B (inset) was densitometrically quantified. The integrated intensity of each band versus time was then drawn (Fig. 3B). It can be observed that 40–60 min after ammonium treatment, when in vivo activity was completely inhibited, the Fe protein labelling was still increasing. This result suggests the existence of another regulatory response of *A. chroococcum* cells to this stimulus that might be faster than, and most likely independent of, the covalent modification of the Fe protein. Alternatively, it can be interpreted in terms that the ethylene production measurement is more accurate than the radioisotope-labelling detection technique used in this study. In connection with these proposals it is worth noting the work by Pierrard et al [11], above referred to, carried out in the phototroph *Rhodopseudomonas capsulatus*, with both the wild-type and mutant strains that cannot be ADP-ribosylated, which have demonstrated the existence of a second response to ammonium that is not accompanied by an inhibition of nitrogenase activity as determined in vitro [11].

3.4. Effect of ammonium inhibition in vivo on nitrogenase activity in situ

In situ assay of nitrogenase activity is carried out in a reconstituted system in which the cell furnishes only the two protein components of the nitrogenase enzyme complex. Cells are permeabilized under anaerobic conditions, supplemented with an artificial electron donor and a readily usable energy source and the reduction of acetylene to ethylene is conveniently followed. The in situ assay allows to distinguish whether or not an in vivo loss of nitrogenase activity is due to a covalent modification as an enzyme modified in such way will not exhibit activity when assayed in situ. By contrast, the presence of in situ activity would reflect an inhibition mechanism that does not imply a permanent modification of the enzyme protein. Between these two extreme cases, situations may be found in which different degrees of covalent modification can be present. The reason for that is the existence of the (Fe protein)-ADP-ribosyl transferase and the (Fe-protein)-activating

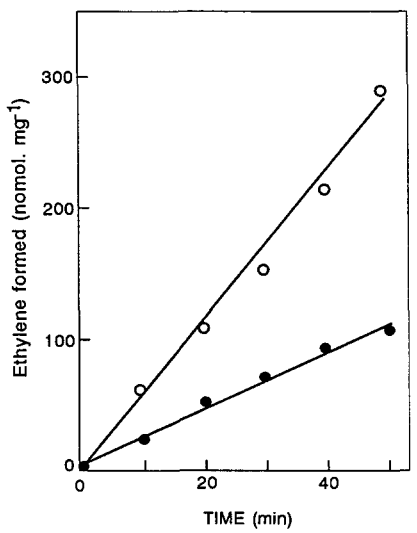

Fig. 4. Effect of NH_4Cl addition to *A. chroococcum* cells suspensions on nitrogenase activity as measured in situ. N_2-fixing bacteria were collected and suspended in 50 mM Hepes/KOH buffer, pH 7.5, containing 1% (w/v) sucrose. Two cell suspension samples were then used to assay the in vivo nitrogenase activity in the absence and in the presence of 5 mM NH_4Cl. After 50 min from the addition of ammonium, the treated cell suspension (●) (containing inactive nitrogenase) and that without additions (○) (containing active nitrogenase) were analyzed for their in situ nitrogenase activity content. Nitrogenase assays as described in Section 2.

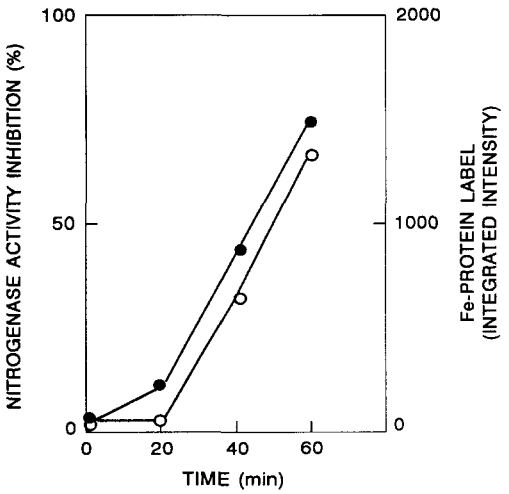

Fig. 5. Comparison of relative ammonium inhibition of in situ nitrogenase activity and covalent modification of the Fe protein component. Fe protein modification values (●) are as in Fig. 3B. In situ activity was assayed at the indicated times after the addition of ammonium. The percentage of nitrogenase activity inhibition (○) was calculated considering 100% the activity of cells non-treated with ammonium and it corresponded to 6 nmol ethylene (mg protein)$^{-1}$ min^{-1}.

glycohydrolase activities in the reaction mixture. Though they require specific reaction conditions to be operative, for example, some cations and metabolites [2], no precautions are usually taken for avoiding these interferring activities.

Fig. 4 depicts the results obtained when bacteria that had been given the ammonium treatment, as well as their untreated control, were also assayed for in situ nitrogenase activity. At 50 min from adding the stimulus, when the nitrogenase activity was in vivo undetectable (see Fig. 3A), the in situ activity had decreased approximately by 60% with respect to its control. This result suggests that most of the Fe protein component of *A. chroococcum* nitrogenase had been covalently modified. The decrease of in situ activity depends on the time after ammonium treatment. Fig. 5 shows that the effect of ammonium on in situ nitrogenase activity is observed only after 20 min. The kinetics of decrease of in situ nitrogenase activity was similar to the kinetics of labelling of the Fe protein. These data therefore show that in situ nitrogenase activity may be taken as an indication of the amount of Fe protein covalently modified. Again, it is observed that in vivo inhibition of nitrogenase activity by ammonium in *A. chroococcum* is faster than the labelling of Fe protein lending support to our proposal that a second regulatory response to ammonium, besides the covalent modification of nitrogenase, exists in this bacterium.

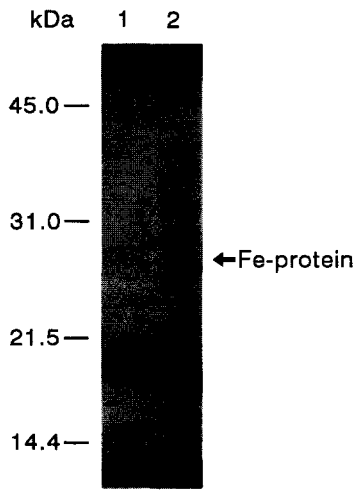

Fig. 6. In vivo $^{32}P_i$ labelling of nitrate-inactivated Fe protein from *A. chroococcum* ATCC 4412 cells. *A. chroococcum* cells grown under nitrogen-fixing conditions, washed and resuspended in N-free medium, were supplied with 5 mM KNO_3 (final concentration) and incubated at 30°C under growth conditions in the presence of [^{32}P]ortophosphoric acid. After 2 h, cells were disrupted and the extracts thus obtained (50 μg of protein) were electrophoresed in an SDS/10%-polyacrylamide gel and the gel subjected to autoradiography. Lanes: 1, cell extract from untreated cells; 2, cell extract from cells incubated in the presence of nitrate.

3.5. Molecular mechanism of the short-term nitrate inhibition of nitrogenase activity in A. chroococcum

In previous work from this group [14], it was found that, in *A. chroococcum* cells simultaneously exhibiting activities of nitrogenase and nitrate assimilation, nitrate exerted a short-term inhibitory effect on nitrogenase activity. We presented evidence supporting that this inhibition was due to some organic product(s) formed during the assimilation of the ammonium resulting from nitrate reduction. We have now examined if nitrate inhibition of nitrogenase activity involves also a covalent modification of the Fe protein. In vivo $^{32}P_i$ labelling of this component in response to nitrate was thereby studied.

N_2-fixing *A. chroococcum* cells, and hence exhibiting high levels of nitrogenase activity, were collected and divided into two fractions: one of them was incubated again under diazotrophic conditions and the other supplemented with 5 mM KNO_3. Both fractions received besides radioactive-labelled phosphoric acid. 2 h later, the nitrate-treated cells displayed the ability to assimilate nitrate and to fix molecular nitrogen, as previously reported [14]. The two cell suspensions were separately collected and their extracts obtained. SDS-PAGE analysis of these preparations revealed incorporation of radioactivity into the Fe protein component of the nitrogenase from nitrate-treated cells, as shown in lane 2 of Fig. 6, whilst only an insignificant incorporation of $^{32}P_i$ took place in cells that did not receive the nitrate treatment (lane 1 in Fig. 6). These results indicated that nitrate inhibition of nitrogenase activity involves a covalent modification of the Fe protein component, most likely induced by the ammonium generated in nitrate assimilation.

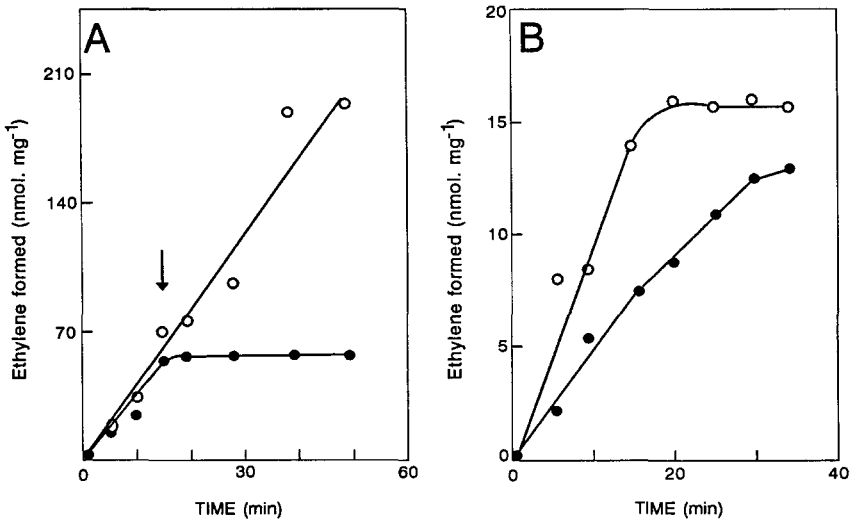

Fig. 7. Effect of KNO_3 addition to *A. chroococcum* cells suspensions on nitrogenase activity as measured in vivo and in situ. Nitrate-grown cells washed and resuspended in N-free medium were incubated at 30°C under growth conditions. After 15 min, cells were collected, resuspended in 50 mM Hepes/KOH buffer, pH 7,5, containing 1% (w/v) sucrose. Two cell suspension samples were then used to assay (A) the in vivo nitrogenase activity in the absence (○) and in the presence of 1 mM KNO_3 (●), which was added when indicated, and (B) the in situ nitrogenase activity after 40 min from the addition of nitrate in the untreated cell suspension (○) (containing active nitrogenase) and in that given the nitrate treatment (●) (containing inactive nitrogenase). Nitrogenase assays as described in Section 2.

3.6. Effect of nitrate inhibition in vivo on nitrogenase activity in situ

As just described, nitrate inhibition of nitrogenase activity resulted in a covalent modification of the Fe protein component. It was expected therefore that nitrate inhibition in vivo would cause a nitrogenase inactivation that should be apparent also in the in situ assay of the enzyme. To verify that this was the case, nitrate-grown *A. chroococcum* cells and hence expressing the nitrate assimilation system only [26] were collected, washed, and resuspended in combined nitrogen-free medium. In agreement with previously described results [14], the nitrate-grown cells diminished their nitrate uptake ability simultaneously with derepression of nitrogenase development when transferred to the combined nitrogen-free medium; after 15–20 min of incubation cells exhibited both nitrate assimilation activity and nitrogenase activity in assayable amounts. As seen in Fig. 7A, addition of 1 mM KNO_3 (final concentration) to such cells promoted a short-term inhibition in vivo of nitrogenase activity. At 45 min from the addition of nitrate the control cells and the nitrate-treated ones were assayed for in situ nitrogenase activity. Fig. 7B illustrates that during the first 10 min of the assay, time period in which the time course of acetylene reduction was linear, in situ nitrogenase activity of the nitrate-treated cells was approximately half of that from untreated bacteria. These data confirm the presence of an inactive, covalently modified nitrogenase enzyme.

Taken together, the results presented in this work strongly support that, in *A. chroococcum*, ammonium, independently of whether exogenously added or intracellularly produced from nitrate assimilation, induces a covalent modification of nitrogenase, most likely an ADP-ribosylation of one subunit of the Fe protein dimer, that has been shown to produce a nitrogenase catalytically inactive [27]. As a consequence, the inactive Fe protein appears as two polypeptide chains with differential migration on SDS/PAGE.

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