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ORIGINAL PAPER

Posttranslational modification of dinitrogenase reductase in *Rhodospirillum rubrum* treated with fluoroacetate

Natalia Akentieva[1]

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Abstract
Nitrogen fixation is one of the major biogeochemical contributions carried out by diazotrophic microorganisms. The goal of this research is study of posttranslational modification of dinitrogenase reductase (Fe protein), the involvement of malate and pyruvate in generation of reductant in *Rhodospirillum rubrum*. A procedure for the isolation of the Fe protein from cell extracts was developed and used to monitor the modification of the Fe protein in vivo. The subunit pattern of the isolated the Fe protein after sodium dodecyl sulfate–polyacrylamide gel electrophoresis was assayed by Western blot analysis. Whole-cell nitrogenase activity was also monitored during the Fe protein modification by gas chromatograpy, using the acetylene reduction assay. It has been shown, that the addition of fluoroacetate, ammonia and darkness resulted in the loss of whole-cell nitrogenase activity and the in vivo modification of the Fe protein. For fluoroacetate, ammonia and darkness, the rate of loss of nitrogenase activity was similar to that for the Fe protein modification. The addition of NADH and reillumination of a culture incubated in the dark resulted in the rapid restoration of nitrogenase activity and the demodification of the Fe protein. Fluoroacetate inhibited the nitrogenase activity of *R. rubrum* and resulted in the modification of the Fe protein in cells, grown on pyruvate or malate as the endogeneous electron source. The nitrogenase activity in draTG mutant (lacking DRAT/DRAG system) decreased after the addition of fluoroacetate, but the Fe protein remained completely unmodified. The results showed that the reduced state of cell, posttranslational modifications of the Fe protein and the DRAT/DRAG system are important for nitrogenase activity and the regulation of nitrogen fixation.

Keywords Dinitrogenase reductase (Fe protein) · Nitrogen fixation · Posttranslational modification · *Rhodospirillum rubrum*

Introduction

Rhodospirillum rubrum ATCC 11170 are of gram-negative purple bacteria, motile, and spiral shaped from the family *Rhodospirillaceae* class alpha-proteobacteria (Heinrich et al. 2016). It is capable of growth under a broad variety of conditions, including aerobically and anaerobically, under the latter conditions using fermentation or photosynthesis for the production of energy. *R. rubrum* is also a nitrogen-fixing bacteria, it can express and regulates nitrogenase, a protein complex that catalyzes the conversion of atmospheric nitrogen to ammonia (Burris 1991). Because of this important quality, *R. rubrum* was chosen by many researchers as a model organism for study of the molecular mechanism and regulation of nitrogen fixation (Tortajada 2017; Teixeira et al. 2008; Selao et al. 2008; Wolfe et al. 2007; Jonsson et al. 2007).

Nitrogen fixation is catalyzed by nitrogenase (EC.1.18.6.1) in all diazotrophs studied. This complex consists of dinitrogenase (MoFe protein) and dinitrogenase reductase (Fe protein), which are both required for enzyme activity. Dinitrogenase reductase (Fe protein) is the obligate electron donor to MoFe protein (Pope et al. 1985). The posttranslational regulation of nitrogenase has been shown first in *R. rubrum* (Nordlund and Noren 1984). It is known now, that in *R. rubrum ATCC 11170* and in some other photosynthetic bacteria, and in some species of *Azospirillum*, the activity of electron carrier-Fe protein, is regulated on the metabolic level in addition to the transcriptional control

✉ Natalia Akentieva
na_aken@icp.ac.ru; na_aken@mail.ru

[1] Department of Kinetics of Chemical and Biological Processes, Institute of Problems of Chemical Physics, Russian Academy of Sciences, Street Academician Semenov, 1., Chernogolovka 142432, Moscow Region, Russia

operating in all diazotrophs studied (Rabouille et al. 2014; Moure et al. 2012). This metabolic control is manifested as a decrease in nitrogenase activity, the ¨switch-off¨ effect. There are two groups of ¨switch-off¨ effectors, those that reflect the fixed nitrogen status and those are related to the energy/redox status. Ammonium, glutamine and asparagine belong to the former group. A change to darkness in phototrophic bacteria or to anaerobic conditions in *A. brasilense* or addition of NAD+ are examples of the latter (Seefeldt et al. 2009; Soliman and Nordlund 1992).

The ¨switch-off¨ effect suggests that dinitrogenase reductase (Fe protein) can be rapidly, reversibly inactivated by mono-ADP ribosylation of one subunit of dinitrogenase reductase (Nordlund and Hogbom 2013). Inhibition of the Fe protein activity (modification) is achieved by NAD+-dependent ADP-ribosylation of arginine 101 and it is catalyzed by dinitrogenase reductase ADP-ribosyltransferase (EC.2.4.2.37, DRAT) in response to energy limitation or nitrogen sufficiency (Kanemoto and Ludden 1984). Upon relief of these negative stimuli, dinitrogenase reductase-activating glycohydrolase (EC.3.2.2.24, DRAG) removes the ADP-ribose moiety, restoring the original, fully active Fe protein (de-modification) (Lowery et al. 1986; Ludden and Burries 1976).

The posttranslational regulation of nitrogenase activity has been found in many diverse nitrogen-fixing bacteria. Although there have been several studies of possible metabolic signals for these stimuli, including the effect of energy, pools of amino acids, purine nucleotides and metal ions, the nature of the signals for the DRAT/DRAG system is still unknown (Seefeldt et al. 2009; Saari et al. 1984; Kanemoto and Ludden 1987; Paul and Ludden 1984; Li et al. 1987; Nordlund and Högland 1986; Nordlund and Noren 1984; Yoch 1979). In *R. rubrum* the proposed changes in the state of the redox/energy status in the cell after adding of ¨switch-off¨ effectors were proposed as a possible model of the regulation DRAT/DRAG system. DRAT and DRAG from *R. rubrum* have been shown to be sensitive to the redox status of the $[Fe_4S_4]1+/2+$ cluster of Fe protein in vivo and in vitro (Noren and Nordlund1994).

It was clearly shown that DRAG has activity with reduced Fe protein. At the same time it was found that DRAT has a specificity for the Fe protein opposite that of DRAG, acting only upon the oxidized form of the Fe protein. The cellular NAD+ concentration has also been suggested as a positive effector for DRAT (Noren and Nordlund 1994). The role of the reactions the tricarboxylic acid cycle (TCA) in metabolizing malate to generate reductant for nitrogenase, NAD(P)H, has been shown early by the inhibition of nitrogenase activity by fluoroacetate (Brostedt et al. 1997). In diazotrophic systems, fluoroacetate is metabolized to fluorocitrate, which is a potent aconitase inhibitor. Inhibition of aconitase activity leads to a decrease in the level of NAD(P)H and is accompanied by the accumulation of NAD+ (Halbleib et al. 2000).

It was shown that nitrogenase activity in *R. rubrum* is dependent on reactions leading to production of NAD(P)H and that these reduced pyridine nucleotides can act as electron donors to the pathway of electron transfer to nitrogenase (Nordlund and Noren 1984; Noren et al. 1997; Brostedt et al. 1997; Ponnuraj et al. 2005). However, the effect of malate and pyruvate as electron donors on nitrogenase activity has not been studied yet. Additionally, no available information about the correlation between the redox status of cell and posttranslational regulation of DRAT/DRAG system.

Therefore, the main aim of this work is to study the involvement of the reactions of TCA cycle in metabolizing malate and pyruvate to generate reductant in *R. rubrum*. Moreover, another idea of this research is investigation the effect of redox status of cell on regulation of DRAT/DRAG system. Based on this, we studied the inhibition of nitrogenase activity by fluoroacetate and monitored modification of the Fe protein by immunoblotting. Effect of different compounds on demodification of the Fe protein was also investigated. The study focuses on the posttranslational regulation of dinitrogenase reductase activity, which can be relevant for deeply understanding the molecular mechanism and regulation of nitrogen fixation.

Materials and methods

Chemicals and media

Unless otherwise specified, all reagents were obtained from Sigma–Aldrich (USA) or GE Healthcare (USA) and were of the highest purity grade available. The following chemicals were used in this study: fluoroacetate (Fac), malate (mal), pyruvate, NAD+ and NADH from Sigma (USA). Reagents for SDS–PAGE and Western blot were purchased from GE Healthcare (USA).

Bacterial growth

Rhodospirillum rubrum, ATCC 11170 (wild-type) and UR212 (draTG mutant) were acquired from Institute Genetics RAS (Moscow, Russia).

Rhodospirillum rubrum, ATCC 11170 (wild-type) and UR212 (draTG mutant) were grown photoheterotrophically on Ormerod medium (Ormerod et al. 1961) with the following modifications: 40 mM D,L-malate or D,L-pyruvate as carbon source, 27 mM L-glutamate, 0.37 mM potassium phosphate, and 52 mM (2-*N* morpholinepropanesulfonic acid) (MOPS) were added. The medium was adjusted to pH 6.7 with NaOH before autoclaving. Cell cultures were grown at 30 °C in water-jacketed fermentor vessels (150 to

500 ml) under an atmosphere of 95% N_2 plus 5% CO_2 at 300 °C, under constant illumination and with constant stirring. Illumination was provided by a 150-W reflector flood lamp located 4 cm from the vessel. *R. rubrum* UR212 cultures were grown in the presence of 12.5 μg kanamycin ml^{-1}.

Detection of the nitrogenase activity and posttranslational modification dinitrogenase reductase in *R. rubrum* after treatment with fluoroacetate, NADH and malate

Cells were grown on 40 mM D,L-pyruvate to OD_{600} 1.0. Then 5 ml of cells were transferred to two of 60 ml vials, which were evacuated and flushed with argon during 10–20 min. After this, fluoroacetate was added to one vial (final concentration 2 mM) and incubated during 180 min. Then, after 80 min of incubation with fluoroacetate, NADH (5 mM) was added to the vial and after 120 min mixture of NADH (10 mM) and malate (40 mM) was added to the vial. Nothing was added to the control vial.

During the all incubation time (180 min) culture samples were withdrawn to measure the nitrogenase activity and Western blot analysis of dinitrogenase reductase. The nitrogenase activity was monitored by gas chromatography (GC), using the acetylene reduction assay (Koch and Evan 1966; Stewart et al. 1967; Ludden and Burris 1978). Triplicate assays were done anaerobically during the incubation period. The reaction was stopped after 2 to 5 min by the addition of trichloroacetic acid [10% (wt/vol.), final], and the ethylene produced was quantitated with a Varian 940 flame ionization gas chromatograph fitted and a Porapak R column (Thermofisher, USA). Nitrogenase activity was expressed as nanomoles of ethylene formed per hour per milliliter of cells assayed. It was assumed that this assay measures all of the active nitrogenase present in vivo. Western blots were developed by using the SuperSignal West Pico Chemiluminescent Substrate System (Thermo Scientific, USA) and the bands were visualized using a LAS-1000 Darkbox II (Fujifilm, Japan) and the MultiGauge v3.0 (Fujifilm, Japan) image analysis software. Each experiment was independently repeated at least three times.

Monitoring of nitrogenase activity and posttranslational modification of the Fe protein after treatment with fluoroacetate and NADH

Posttranslational modification of the Fe protein after treatment with fluoroacetate carried out according to method, described earlier (Brostedt et al. 1997; Elsden and Ormerod 1956). Shortly, cells *R. rubrum* were grown with 40 mM D,L-malate to OD_{600} 1.0. Then 5 ml of cells were transferred to two of 60 ml vials, which were evacuated and flushed with argon during 10–20 min. After this, fluoroacetate (final concentration 2 mM) was added to one vial and incubated during 120 min. Then after 105 min, NADH (5 mM) added to the vial. Nothing was added to the control vial. During incubation time the nitrogenase activity was measured by acetylene reduction and culture samples were withdrawn for Western blot analysis of the Fe protein from both vials (Stewart et al. 1967; Ludden and Burris 1978). Each experiment was independently repeated at least three times.

The measurement of posttranslational modifications of dinitrogenase reductase after treatment with fluoroacetate and additions of NADH, malate, dark and light

Cells *R. rubrum* were grown with 40 mM D,L-malate to OD_{600} 1.0. Then 5 ml of cells were transferred to two of 60 ml vials, which were evacuated and flushed with argon during 10–20 min. After this, fluoroacetate (final concentration 2 mM) was added to one vial and incubated during 300 min. Then after 103 min, NADH (5 mM) added to the vial. After 118 min D,L-malate (40 mM) was added and vial incubated up to 208 min. Nothing was added to the control vial. Then first vial was transferred to dark (wrapped in three layers of with aluminium foil) and incubated during 30 min. After this, vial was again transferred to light. During the incubation time (300 min), at indicated time intervals, culture samples were withdrawn to measure the nitrogenase activity and Western blot analysis of dinitrogenase reductase. The data are presented as averages from three repeated experiments.

The effect of fluoroacetate and ammonium on nitrogenase activity in *R. rubrum*

Cells were grown with 40 mM D,L-malate to OD_{600} 1.0. Then 5 ml of cells were transferred to two of 60 ml vials, which were evacuated and flushed with argon during 10–20 min. After this, fluoroacetate (final concentration 2 mM) was added to one vial and incubated during 140 min. After 95 min ammonium chloride (0.25 mM) was added. During incubation time the nitrogenase activity was measured by acetylene reduction and culture samples were withdrawn for Western blot analysis of the Fe protein. Each experiment was independently repeated at least three times.

The effect of fluoroacetate and NADH at the nitrogenase activity and modification of the Fe protein in UR212 (draTG mutant)

Culture of UR212 (draTG mutant) mutant were grown under nitrogen-fixing conditions with 40 mM D,L-malate. Cells were grown to OD_{600} 1.0. Then 5 ml of cells were transferred to two of 60 ml vials, which were evacuated and flushed with argon during 10–20 min. After this, fluoroacetate (final

concentration 2 mM) was added to one vial and incubated during 160 min. Then after 110 min NADH (5 mM) was added. During incubation time the nitrogenase activity was measured by acetylene reduction and culture samples were withdrawn for Western blot analysis of the Fe protein. As a control we used the modified Fe protein in wild-type of *R. rubrum*. The data are presented as averages from three repeated experiments.

Western blot analysis of dinitrogenase reductase

To monitor the modification state of dinitrogenase reductase during fluoroacetate treatment conditions, culture samples were processed and analyzed by gel electrophoresis. From each vessel the 0.25 ml of cell culture were withdrawn anaerobically and injected into a 21-ml vial, containing 0.25 ml of anaerobic sodium dodecyl sulphate (SDS) buffer. The SDS buffer contained 130 mM Tris (pH 6.8), 4.2% (wt/vol.) SDS, 20% (vol./vol.) glycerol, 0.003% (wt/vol.) bromphenol blue, and 10% (vol./vol.) 2-mercaptoethanol (added fresh). The vials were incubated at 300 °C for 1 min and then boiled for 1 min. Then the vials were centrifuged at 14,000*g* during 10 min, and supernatants were withdrawn and stored at − 200 °C.

Culture samples, prepared as described above, were analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) with low-cross link SDS–PAGE gels [10% (wt/vol.) with a ratio of acrylamide to bisacrylamide, 172:1], which separate the ADP-ribosylated and unmodified subunits of dinitrogenase reductase (Laemmli 1970; Kanemoto and Ludden 1984). Proteins, separated by SDS–PAGE, were electrophoretically transferred onto a PVDF membrane. Western blot analysis was performed by using primary antibodies raised against *R. rubrum* dinitrogenase reductase at a 1:10,000 dilution at 300 °C overnight. The blot was further incubated with secondary anti-rabbit antibodies, horseradish peroxidase linked F (ab') 2 fragment (from donkey). Blots were developed by using the SuperSignal West Pico Chemiluminescent Substrate System and the bands of dinitrogenase reductase were visualized by ECL detection, using a LAS-1000 Darkbox. Each experiment was independently repeated at least three times.

Nitrogenase activity

Rhodospirillum rubrum cells from exponentially growing cultures were centrifuged and inoculated into sealed vials containing fresh medium and grown for 48 h in the above-mentioned conditions. Cultures were at OD_{600} of 1.0. at the start of activity measurements. In vivo whole cell nitrogenase activity was measured by using the acetylene reduction assay and each experiment was independently repeated at least three times (Koch and Evan 1966; Stewart et al. 1967; Ludden and Burris 1978). The ethylene produced was quantitated with a Varian 940 flame ionization gas chromatograph fitted and a Porapak R column (Thermofisher, USA). Nitrogenase activities are expressed as nanomoles of ethylene formed per ml of cell culture per hour, normalized to a cell culture OD_{600} of 1.0.

Results

Inactivation of the Fe protein in cells treated with fluoroacetate and effect electron donors on the level of nitrogenase activity

Rhodospirillum rubrum system was used with the parameters described above. The functional activity of the Fe protein was monitored in response to exogeneous fluoroacetate in vivo. In response to the addition of fluoroacetate (2 mM), of wild type *R. rubrum*, grown on pyruvate, exhibited a loss of nitrogenase activity in 30 min (Fig. 1, Fac treated curve). As a control, cells grew in linear proportion without fluoroacetate (Fig. 1, Control curve).

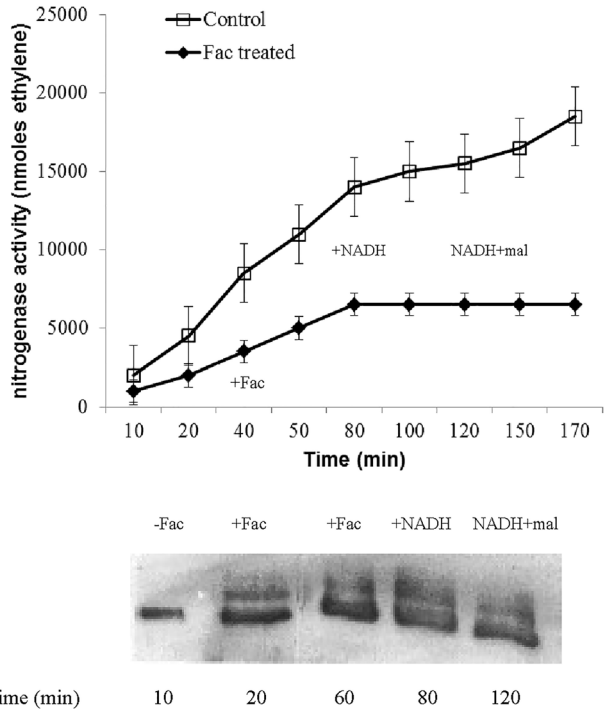

Fig. 1 Response of the nitrogenase enzyme in *R. rubrum* to treatment with fluoroacetate. Cells were grown in linear proportion on 40 mM pyruvate without addition of fluoroacetate (Control curve). The effect of NADH, NADH+ malate on nitrogenase activity in cells treated with fluoroacetate. At times indicated with numbers the following additions were made (Fac treated curve): (1) Fac-2 mM, (2) NADH-5 mM, (3) NADH (10 mM) + malate (40 mM); the bottom panel: corresponding immunoblot analysis of the Fe protein (from Fac treated curve)

Cells, which were grown on malate as carbon source, also showed a decrease of nitrogenase activity after addition of 2 mM fluoroacetate (Fig. 2, Fac treated curve). In comparison, cells grew in linear proportion without fluoroacetate (Fig. 2, Control curve). Addition of NADH resulted in to restoration of nitrogenase activity (Fig. 2, Fac treated curve). Besides of this, NADH increased of nitrogenase activity in approximately 1.5-fold (Fig. 2, Fac treated curve). Neither succinate, pyruvate or hydrogen were restored nitrogenase activity (Data not shown). These data suggest that after treatment with fluoroacetate the level of NAD(P)H decreased and nitrogenase activity was inhibited. But adding a new amount of NADH restored and increased the nitrogenase activity. These results confirm that purine nucleotides are metabolic signals to stimulate nitrogen fixation.

We are also studied response of the nitrogenase activity in *R. rubrum* to fluoroacetate, NADH, malate, exposure to dark and light in cells grown on malate (Fig. 3). Addition of NADH to cells, treated with fluoroacetate, resulted in

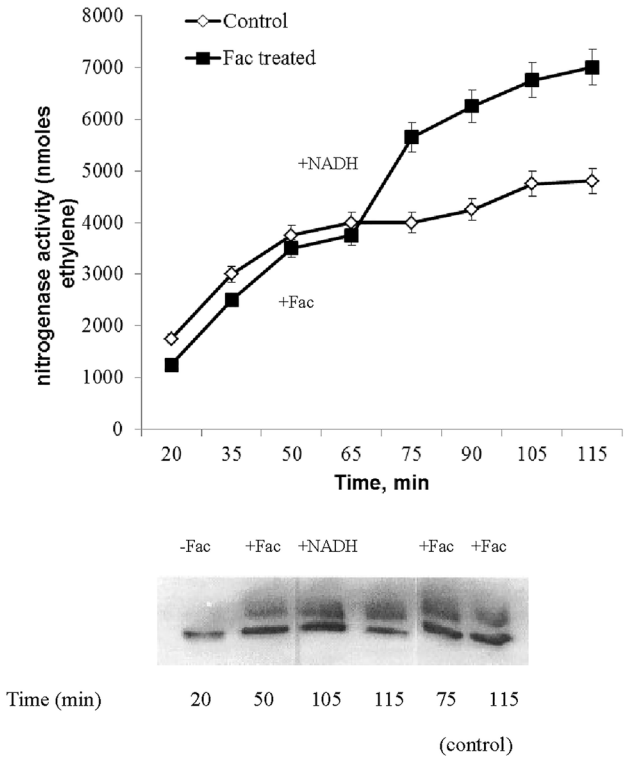

Fig. 2 Correlation of nitrogenase activity with modification of the Fe protein. Cells were grown in linear proportion on 40 mM malate without addition of fluoroacetate (Control curve). The effect of NADH on nitrogenase activity in cells of *R. rubrum* treated with fluoroacetate. At times indicated numbers the following additions were made (Fac treated curve): (1) Fac-2 mM, (2) NADH-5 mM. The bottom panel: corresponding immunoblot of the Fe protein (from Fac treated curve). The upper band corresponds to the ADP-ribosylated form of the Fe protein. The lower band represents unmodified Fe protein. Control: samples without NADH

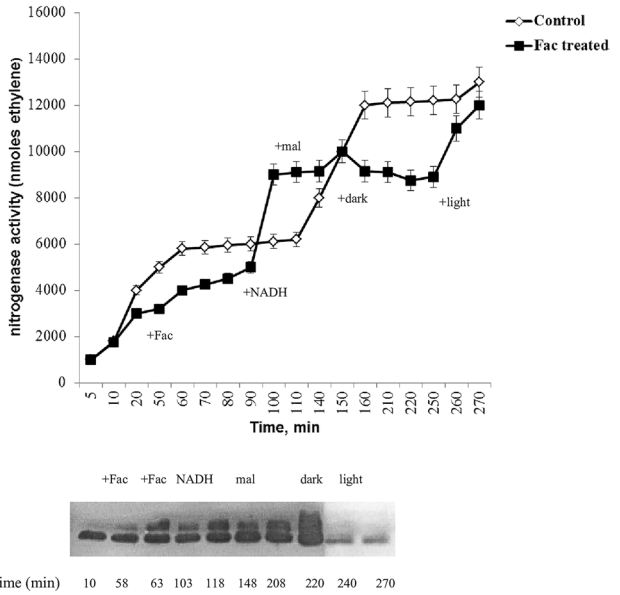

Fig. 3 Effect of NADH, malate, dark and light in cells *R. rubrum* treated with fluoroacetate. Cells were grown in linear proportion on 40 mM malate without addition of fluoroacetate (Control curve). At times indicated the following additions were made (Fac treated curve): (1) Fac-2 mM, (2) NADH-5 mM, (3) malate-40 mM, (4) dark, (5) light. The bottom panel: corresponding immunoblot analysis of the Fe protein (from Fac treated curve)

enhancement of nitrogenase activity in approximately 1.5-fold, but malate did not affect at nitrogenase activity (Fig. 3, Fac treated curve, addition NADH and malate, respectively). However, subjecting cells to darkness reduced the level of nitrogenase activity, but light restored the nitrogenase activity (Fig. 3, Fac treated curve, addition dark and light).

Western blot analysis showed that exposure of cells in the dark led to a strong modification of the Fe protein compared to the effect of fluoroacetate (Fig. 3, the bottom panel, 63 and 220 min). These data suggest that darkness induces the posttranslational modification of the Fe protein. This indicates that darkness is a stronger metabolic signal compared to fluoroacetate.

Our results also showed that ammonium is strong "switch-off" effector of the nitrogenase activity. The addition of ammonium (0.25 mM) to the cells leads to a fast decrease of the nitrogenase activity and an additional modification of the Fe protein (Fig. 4, Fac treated curve). As a control, cells grew in a linear proportion without the addition of fluoroacetate and ammonium (Fig. 4, Control curve).

The Western blot analysis showed an intense upper band of the Fe protein after 110 min (Fig. 4, the bottom panel). However, this modification caused by ammonium was reversible. After 130 min, cells metabolized with ammonium and de-modification of the Fe protein was observed, since the upper band almost completely disappeared (Fig. 4, the bottom panel, 130 min). In addition, after the utilization

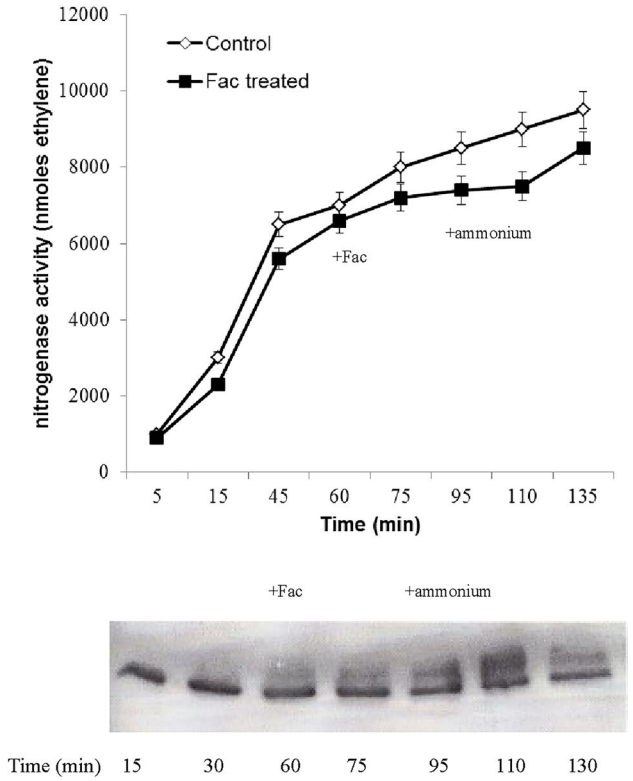

Fig. 4 Effect of fluoroacetate and ammonium on nitrogenase activity in *R. rubrum*. Cells were grown in linear proportion on 40 mM malate without addition of fluoroacetate (Control curve). At times indicated numbers the following additions were made (Fac treated curve): (1) Fac-2 mM, (2) ammonium chloride-0.25 mM. The bottom panel: corresponding immunoblot analysis of the Fe protein (from Fac treated curve)

of ammonium, the level of activity of nitrogenase increased (Fig. 4, Fac treated curve).

Correlation of immunoblots with nitrogenase activity

Nitrogen-fixing cultures of *R. rubrum*, wild-type, were treated with a fluoroacetate (2 mM), and the state of the Fe protein was monitored using Western blot analysis. Immunoblot analysis showed a clear modification of the Fe protein after the addition of fluoroacetate.

Initially, wild type of the Fe protein was unmodified (Fig. 1, the bottom panel, 10 min; Fig. 2, the bottom panel, 20 min). On the immunoblot there is one line of the unmodified subunit of the Fe protein. But after adding of fluoroacetate, a clear modification of the Fe protein the ADP-ribosylated (inactive) state was observed on immunoblot (Fig. 1, 2, 3, 4, the bottom panels, addition Fac) and correlated with a decrease in the activity of nitrogenase (Figs. 1, 2, 3, 4, the top panels).

NADH or NADH+, malate did not restore the nitrogenase activity, and as seen on the immunoblot, the upper band of the modified subunit of the Fe protein did not disappear (Fig. 1, the bottom panel, 80 and 120 min; Fig. 2, the bottom panel, 105 min; Fig. 3, the bottom panel, 103 and 118 min).

Subjecting the cells in the dark increased the modification of the Fe protein, and the intensity of the upper band became stronger (Fig. 3, the bottom panel, 220 min). These data suggest, that darkness induces posttranslational modification of the Fe protein, and the darkness is a stronger "switch-off" effector than fluoroacetate.

Placing cells into light caused a de-modification of the Fe protein, and the upper band disappeared (Fig. 3, the bottom panel, 240 and 260 min). Moreover, the addition of ammonium (0.25 mM) to the cells treated with fluoroacetate also increased the modification of the Fe protein, and the intensity of the upper band became stronger (Fig. 4, the bottom panel, 110 min), but then it decreased after 130 min due to for the utilization of ammonium cells, and the upper band of the Fe protein is almost completely disappeared (Fig. 4, the bottom panel, 130 min). These data indicated that the ammonium effect is reversible.

Inactivation response in UR212 (draTG mutant)

Rhodospirillum rubrum strain containing mutations in draTG (lacking DRAT/DRAG system) was treated with fluoroacetate in order to examine the importance of the effect of DRAT on the rate of ADP-ribosylation. The mutant culture of UR212 was grown under nitrogen-fixing conditions. Nitrogenase inactivation was initiated by the addition of fluoroacetate (2 mM) to the culture. The extent of modification of the Fe protein was investigated by immunoblotting.

The nitrogenase activity of UR212 (draTG mutant) was reduced after the addition of fluoroacetate (Fig. 5, Fac treated curve). For comparison, cells were grown without the addition of fluoroacetate (Fig. 5, Control curve). However, immunoblot data showed that the Fe protein in UR212 (draTG mutant), treated with fluoroacetate, remained completely unmodified for a long period of time (Fig. 5, the bottom panel, 20–150 min). The addition of NADH to these cells did not affect at the level of nitrogenase activity and the modification of the Fe protein (Fig. 5, Fac treated curve, addition NADH). For comparison, the Fe protein was easily modified in wild-type of *R. rubrum* treated with fluoroacetate (Fig. 5, the bottom panel, C).

These data indicate that function of DRAT/DRAG system is important for posttranslational modification of the Fe protein and regulation of nitrogen fixation.

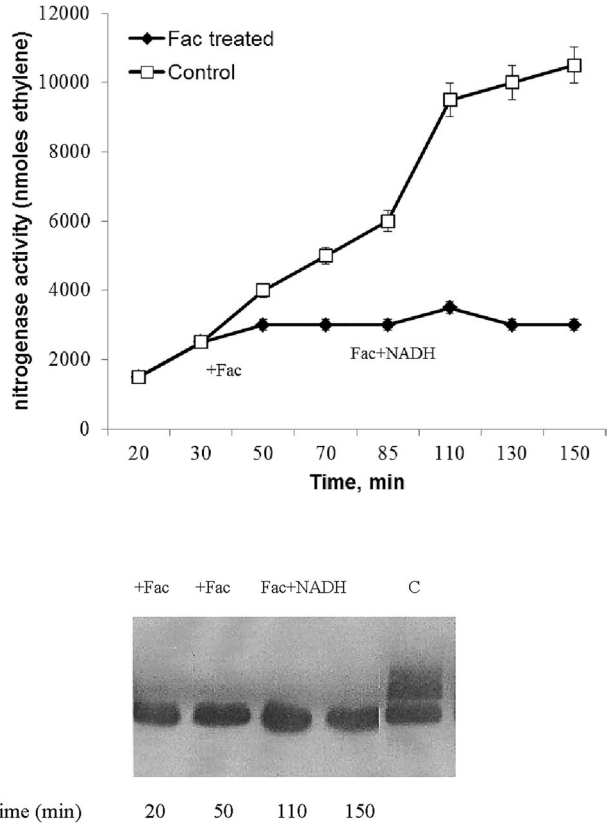

Fig. 5 The effect of fluoroacetate on nitrogenase activity in cells UR212 (draTG mutant) of *R. rubrum*. Cells were grown in linear proportion on 40 mM malate without addition of fluoroacetate (Control curve). Nitrogenase activity monitored after addition of fluoroacetate to Fac treated curve: (1) Fac-2 mM, (2) NADH-5 mM. The bottom panel: Western immunoblot of the Fe protein in draTG mutant from Fac treated curve. (C)-a modified Fe protein in wild-type of *R. rubrum*

Discussion

It has been shown earlier, that all the enzymes of TCA are present in dried-cell preparations of *R. rubrum* (Eisenberg 1953). It has been suggested that generation of reductant to nitrogenase is dependent on the TCA cycle, which has been previously demonstrated in *R. rubrum* (Elsden and Ormerod 1956; Teixeira et al. 2010; Selao et al. 2011; Gorell and Uffen 1978; Luderitz and Klemme 1977). In order to study the involvement of the reactions the TCA cycle in metabolising malate and pyruvate to generate reductant, we investigated the inhibition of nitrogenase activity by fluoroacetate. In this paper, we have shown that addition of fluoroacetate to *R. rubrum* decreased the concentration of reduced NAD(P), which is in agreement with an earlier study on changes of the pyridine nucleotides pools in *R. rubrum* upon change from light to darkness (Elsden and Ormerod 1956; Luderitz and Klemme 1977). These authors showed that in the light about 80% of the NAD(P) pool was in the reduced form, whereas in the dark about 80% was detected as NAD(P)+. It is known, that in *R. rubrum* as in other systems, fluoroacetate is metabolized to fluorocitrate, which is a potent inhibitor of aconitase (Elsden and Ormerod 1956; Jackson and Crofts 1968; Gest et al. 1962). Inhibition of aconitase activity leads to decrease of NAD(P)H level and it is accompanied by an accumulation of NAD+ (Gest et al. 1962).

More than 30 years ago it was demonstrated that in some organisms, such as *R. rubrum, Rhodobacter capsulatus* and *Azospirillum brasilense*, nitrogenase activity is also regulated by reversible ADP-ribosylation of the Fe protein (Nordlund and Noren 1984). The addition and removal of the ADP-ribose moiety in *R. rubrum* are catalysed by two enzymes, dinitrogenase reductase ADP-ribosyltransferase (DRAT) and dinitrogenase reductase activating glycohydrolase (DRAG), respectively (Nordlund and Noren 1984). It has been shown earlier, that in *R. rubrum*, the Fe protein is ADP-ribosylated in response to metabolic stimuli such as darkness or addition of ammonium, so-called "switch-off" effectors (Kanemoto and Ludden 1984). Moreover, it has been demonstrated that the different effects of darkness, with respect to the presence or absence of pyruvate, are due to the activity of the DRAT/DRAG system (Selao et al. 2011). It is reported that DraT is a monomeric 30 kDa protein that catalyses ADP-ribosylation, with NAD+ as the donor of the ADP-ribose moiety (Lowery et al. 1986). In addition, DraT is specific for the Fe protein as an acceptor in *R. rubrum* (Grunwald et al. 2000).

Here we have shown that the main effect caused by fluoroacetate is a decrease of the nitrogenase activity and modification of the Fe protein, ¨switch-off¨ effect. We suppose that this effect increased the rate of the reaction catalysed by DRAT, due to higher substrate availability and increasing in amount of NAD+.

To establish that the effect of fluoroacetate on nitrogenase is mediated through the DRAT/DRAG system, the cultures of *R. rubrum* UR212 were subjected to the same treatment as the wild type cultures. Interestingly, that the UR212 strain lacking both DRAT and DRAG enzymes leads to loss of posttranslational regulation of nitrogenase (Liang et al. 1991). Our results demonstrated that nitrogenase activity in mutant UR212 decreased after addition of fluoroacetate. However, Western blot results did not show the modification of the Fe protein in case of using a mutant of *R. rubrum*, lacking DRAT/DRAG system. These results indicate that the posttranslational modifications of the Fe protein are due to the activity of the DRAT/DRAG system. Furthermore it suggests that the effect of fluoroacetate on nitrogenase activity is also due to this system.

We have observed the inhibition of the Fe protein activity by fluoroacetate in cells, grown with malate or pyruvate as the endogeneous electron source. It indicates that these compounds have to be metabolized via the TCA cycle in

order to support nitrogenase activity. Previously it has been shown that reduced pyridine nucleotides can support electron transfer to nitrogenase and that the activity is concentration dependent (Soliman and Nordlund 1992; Li et al. 1987; Nordlund and Högland 1986; Noren et al. 1994; Noren and Nordlund 1997; Brostedt et al. 1997; Ponnuraj et al. 2005). It has been demonstrated also that the sensitivity of the DRAT and DRAG regulatory enzymes depends from the redox state of the substrate, the Fe protein (Noren and Nordlund 1994; Halbleib et al. 2000; Kanemoto and Ludden 1984; Gorell and Uffen 1978; Gest et al. 1962). It should be noted that our results showed a restoration of nitrogenase activity after addition NADH to cells, grown on malate and treated by fluoroacetate. At the same time, we have demonstrated that pyruvate, succinate or hydrogen did not influence on nitrogenase activity. However, we have observed that after addition of NADH to cells treated with fluoroacetate nitrogenase activity enhanced in approximately 1.5-fold. Thus, these results confirm that pyridine nucleotides are significant for nitrogenase activity. These data indicate also, that redox state of cell regulates and effects on DRAT/DRAG activity.

In this paper we have also shown that addition of fluoroacetate correlates with the ADP-ribosylation of the Fe protein, but there is appear quite a lag between addition of fluoroacetate and decrease in nitrogenase activity. It is interesting that the length of the lag is approximately 30 min. Probably, this time is needed for conversion of fluoroacetate in fluorocitrate, inhibition of aconitase, accumulation NAD+ and activation of DRAT enzyme. As shown at Fig. 1, at first the nitrogenase activity slowly decreases, and after 80 min it decreases several times in comparison with the control, but then the activity of nitrogen stabilizes. This lag time is quite significant for the cells, because during this time the DRAT enzyme is activated and the modification of the Fe protein is observed. In addition, in cells treated with fluoroacetate during the entire experimental time, we observed two bands of the Fe protein, a modified and unmodified form. This suggests that DRAG and DRAT are simultaneously active, probably because a mixture of reduced and oxidized Fe protein may be present at any time. Thus, the addition of fluoroacetate leads to the half-modification of the Fe protein, since the intensity of the upper band was lower, comparable to the lower band. These results definitely showed that fluoroacetate leads to a more oxidized state of the cell and activation of the DRAT enzyme. However, modification of the Fe protein after addition of fluoroacetate was incomplete (about 50%) and complete inactivation of the nitrogenase activity was achieved only after the addition of ammonium.

We have shown in this paper that DRAT is required for modification of the Fe protein in *R. rubrum*, using the UR212 strain (draTG mutant). However, it may be possible, that the regulation of the Fe protein by DRAG/DRAT system involves additional protein in vivo. It has been suggested earlier that a protein AmtB, an ammonium transporter, can be such partner for DRAT (Huergo et al. 2007; Javelle et al. 2008). Furthermore, it is generally believed that a PII protein, (GlnJ in *R. rubrum* and GlnZ in *A. brasilense*) plays a central role in the process leading to association of DRAG with the membrane in response to ammonium ions as the effector. Recent studies have proposed a model of the regulation of DRAT and DRAG, preferably in complex with a PII protein (Moure at al. 2012).

Although the modification and enzymes catalysing the modification of the Fe protein are known, many details regarding the role and regulation of DRAT and DRAG are yet to be clarified, especially in response to darkness. Here it is shown that the addition of malate does not significantly affect the level of nitrogenase activity. However, in response to a strong negative stimulus, such as darkness or the addition of ammonium, we observed an increase in the activity of DRAT with a subsequent inhibition of the activity of nitrogenase. But putting cells into darkness and then returning them to light again increased the activity of nitrogenase. In addition, the immunoblot showed a strong modification of the Fe protein in the dark and the disappearance of the upper band of modified Fe protein after placing the cells on the light. Strong negative "switch-off" effectors, such as darkness and ammonium, lead to a complete modification of the Fe protein. This indicates that the reduced state of the cell is very important for nitrogen activity. It is possible that local changes in the redox potential, as well as variations in the ATP/ADP ratios and NAD+/NADH concentrations, may lead to the dramatic regulation observed in DRAT and DRAG in vivo. This is consistent with a regulatory model where the redox state of the cell is important.

We have described in this paper a method for the analysis of the Fe protein by Western blot and for the detection of its modification state. This procedure has enabled the observation of the Fe protein inactivation and activation in vivo. We have observed that the reduction of whole-cell nitrogenase activity upon ammonium treatment was correlated with the modification of active Fe protein in vivo. This is the first demonstration of the rate of the Fe protein modification in vivo in *R. rubrum* during ammonia "switch-off". The molecular basis of this response of nitrogenase to ammonium is unknown. We can suggest a role for glutamine synthetase (GS) or glutamine pools in the ammonium "switch-off" effect. A role for GS or glutamine pools in NH4+-dependent inhibition of nitrogenase activity in photosynthetic bacteria has been suggested by several researchers (Lowery and Ludden 1988).

Thus, in this article, we showed for the first time that the loss of cellular nitrogenase activity due to the addition of ammonia, darkness, and fluoroacetate correlates with the in vivo change state of the Fe protein. We have also showed rapid activation of modified Fe protein in vivo by

light. Several treatments with dark-light have demonstrated dynamic regulation of nitrogenase activity and the Fe protein modification. Previously, Pratt and Schick demonstrated the importance of light for the activity of nitrogenase in vivo (Pratt and Frenkel 1959; Schick 1971).

Here we confirmed that cells could not maintain the nitrogenase activity in the dark, and we found that dark conditions caused a modification of the Fe protein similar to that observed with the addition of ammonia.

The re-illumination of a dark "switched-off" culture led to the rapid recovery of nitrogenase activity and the removal of the phosphate and adenine components of the modifying group from the Fe protein. These observations indicate that light has some influence on the regulation of cellular nitrogenase activity in addition to providing energy for MgATP synthesis or reductant generation (Rabouille et al. 2014). It should be noted, that the experimental advantage of the inactivation and activation of *R. rubrum* of the Fe protein in vivo by the use of dark-light treatment is that light can be rapidly supplied to the cells and then removed with no residual effect.

Therefore, our results showed that malate and pyruvate capable to generating the reducing agent in *R. rubrum*, the redox status of cell is important for regulating the DRAT/DRAG system, which is responsible for posttranslational modification of the Fe protein.

Further studies are required to clarify the regulatory mechanisms in *R. rubrum* and in other diazotrophs.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict interests.

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