The Oxygen Sensitivity of Nitrogenase in *Rhodobacter capsulatus*: Repression and Inactivation

Gesine Klein[a], Andreas Jahn[a], Astrid Steindorf[a], Werner Klipp[b], Jürgen Oelze[a]

[a] Institut für Biologie II (Mikrobiologie), Universität Freiburg, Schänzlestraße 1, D-79104 Freiburg, Bundesrepublik Deutschland

[b] Biologie VI (Genetik), Universität Bielefeld, Postfach 100131, D-33501 Bielefeld, Bundesrepublik Deutschland

Z. Naturforsch. **51c,** 20–28 (1996); received October 30, 1995

Nitrogenase, Oxygen Control, Chemostat Culture, Phototrophic Bacteria, *Rhodobacter capsulatus*

In chemostat cultures of *Rhodobacter capsulatus*, growing aerobically in darkness, *in situ* nitrogen fixation occurred at significantly lower oxygen concentrations than the acetylene reduction activity of nitrogenase, as determined with samples from the culture under optimum assay conditions. In contrast to nitrogenase of cultures growing diazotrophically at low oxygen, nitrogenase present in inactive form at the higher oxygen concentrations could not be activated to fix nitrogen by increasing i.) the energy supply by illuminating the cultures, ii.) the supply of cells with electron donor and iii.) cellular respiration. These results suggest that oxygen controls cellular nitrogen fixation directly rather than indirectly by interfering with the general metabolism. On this basis, the sensitivity toward oxygen of *nif* gene expression as well as of nitrogenase polypeptide accumulation was studied. The *nifH* promoter was active up to about 40% air saturation, exhibiting a biphasic sensitivity to oxygen, i.e. steep decreases in activity between 1 to 2% and between 10 to about 20% air saturation. A similar behaviour was observed with respect to cellular levels of both polypeptides of component 1 (Rc1) of nitrogenase, which were accumulated up to about 20% air saturation. Component 2 (Rc2) was less oxygen-sensitive than Rc1, its steady state level reached a maximum at 2% air saturation. A sudden increase in the ambient oxygen concentration, i. e. oxygen stress, revealed that, *in vivo,* nitrogenase was fairly stable against oxygen damage. Modification of Rc2 by ADP-ribosylation occurred under ammonium shock conditions, regardless of whether the cells fixed nitrogen or not. But it was not observed with cultures growing aerobically in the dark at steady state or under oxygen stress. Results obtained with mutant W107I of *R. capsulatus* lacking the modifying/demodifying enzymes supported the conclusion that modification of Rc2 was not required for the inactivation of nitrogenase by oxygen.

Introduction

The biological reduction of dinitrogen to ammonia is catalyzed by the nitrogenase enzyme complex confined to prokaryotes. The classical nitrogenase is composed of two functional subunits, the molybdenum iron protein or component 1 and the iron protein or component 2 (Burris, 1991). Component 1 contains two copies of each, an α- and a β-polypeptide, while component 2 is made up of two copies of the *nifH* gene product. *In vitro*, both subunits of nitrogenase are rapidly and irreversibly inactivated by oxygen (Robson and Postgate, 1980). *In vivo*, oxygen represses the transcription of *nif* genes, encoding the nitrogenase polypeptides and other products required for diazotrophic growth (Gussin *et al.*, 1986; Kranz and Foster-Hartnett, 1990; Foster-Hartnett and Kranz, 1992; Gallon, 1992). Moreover, a sudden increase in the oxygen concentration i. e. oxygen stress, reversibly inactivates nitrogenase *in vivo* (Ludden and Roberts, 1995). Nevertheless, the phototrophic *Rhodobacter capsulatus* is known to fix nitrogen and, thus, to grow diazotrophically if exposed to very low oxygen concentrations (Siefert and Pfennig, 1980). Recently, it was shown that *R. capsulatus* is able to grow diazotrophically only at oxygen concentrations below 2% air saturation. Yet, the nitrogenase complex is present in cells adapted to oxygen concentrations up to about 30% air saturation (Klein *et al.*, 1993).

Molecular genetic studies on the arrangement of *nif* genes revealed that genes encoding the three nitrogenase polypeptides NifH (component 2) and NifDK (component 1) are transcribed

Reprint requests to Prof. Oelze.
Fax: +49 (0)761-2032773.

0939–5075/96/0100–0020 $ 06.00

from a common promoter (Gussin *et al.*, 1986; Willison *et al.*, 1993). A transcriptional regulator protein, NifA, necessary for the activation of the *nifHDK* promoter, is subject to oxygen-inactivation (Fischer and Hennecke, 1987; Benyon *et al.*, 1988). In *R. capsulatus*, however, control of *nif* transcription may become more complicated because this organism harbours two independent copies of *nifA*, transcription of which showed different sensitivities toward ammonia and oxygen (Masepohl *et al.*, 1988; Foster-Hartnett and Kranz, 1992; Preker *et al.*, 1992). In addition, oxygen repression of *nif* gene transcription was suggested to be regulated at the level of DNA supercoiling (Kranz and Haselkorn, 1986). At the moment neither the concentrations of oxygen, controlling different levels of nitrogenase expression, nor the influence of this control are known.

Reversible inactivation of nitrogenase taking place not only under oxygen stress but also upon addition of a source of fixed nitrogen or upon transfer of cells from light to darkness, was studied mainly with *Rhodospirillum rubrum* and to a lesser extent with *R. capsulatus*. The results led to the hypothesis that electron transport from component 2 to component 1 becomes inhibited by a reversible ADP-ribosylation of component 2 (Ludden and Roberts, 1995). However, the fast inactivation of nitrogenase by oxygen was reported to be accompanied by only an incomplete modification of component 2 of *R. rubrum* (Kanemoto and Ludden, 1984). Lack of a strict dependency of inactivation of component 2 (Rc2 = NifH, Rc1 refers to component 1 or the NifDK polypeptides of *R. capsulatus*) on modification by ADP-ribosylation has been supported by more recent studies performed with *R. capsulatus* subjected to ammonium shock (Pierrard *et al.*, 1993). This suggests the presence of additional mechanisms involved in the inactivation of nitrogenase. In addition to the possibility of entering an inactive form, the *in vivo* function of nitrogenase may be inactivated due to the lack of reducing equivalents or energy.

The present study has been performed in order to define the oxygen concentrations controlling repression as well as reversible inactivation of nitrogenase *in vivo*.

Materials and Methods

Bacterial strains and growth conditions

The experiments were performed with the following strains of *Rhodobacter capsulatus*: the wild-type strain B10, strain B10S W107I lacking both the modifying (DraT) and the demodifying (DraG) enzymes of Rc2 (Masepohl *et al.*, 1993) and strain R372 carrying a chromosomal *nifH-lacZ* transcriptional fusion. The organisms were grown in chemostat culture in the dark at defined oxygen concentrations as previously described (Klein *et al.*, 1993). Oxygen concentrations are given as percent air saturation of the medium, where 100 % air saturation corresponds to 230 μM dissolved oxygen (Post *et al.*, 1982). The growth medium was RCVBN (Weaver *et al.*, 1975). In order to limit the relative supply of bound nitrogen, ammonium sulfate and D,L-malate were added to cultures of the wild-type and the mutant strain W107I at concentrations of 0.5 mM and 25 mM, respectively. In order to obtain samples with higher cell densities for β-galactosidase assays, cultures of the *nifH-lacZ*-fusion mutant of *R. capsulatus* were supplied with three-fold higher concentrations of ammonium sulfate and D,L-malate.

Construction of a R. capsulatus strain carrying a nifH-lacZ fusion

For the construction of a chromosomal *nifH-lacZ* fusion, a 7 kb DNA fragment encompassing the entire *nifHDK* operon of *R. capsulatus* was cloned into a mobilizable vector plasmid, which is unable to replicate in *R. capsulatus*. Subsequently, a 4 kb BglII-HindIII fragment harbouring the 3' end of *nifH*, *nifD* and *nifK*, was substituted by a cassette carrying the promoterless *lacZYA* operon of *Escherichia coli* and a constitutively expressed tetracycline resistance gene. This cassette was obtained from plasmid pML5B$^+$ (Labes *et al.*, 1990). The resulting *nifH-lacZ* fusion plasmid (pWKR372) was conjugationally transferred from *E. coli* S17–1 to *R. capsulatus* as described (Masepohl *et al.*, 1988) and double cross-over recombination of the *lacZYA-Tc^r* cassette was selected by tetracycline resistance and loss of the vector encoded spectinomycin resistance. Correct cross-over was verified by Southern hybridization.

Determination of cellular activities

Nitrogenase was determined on the basis of either *in situ* nitrogen fixation or acetylene reduction. *In situ* nitrogen fixation, i.e. nitrogen fixation by the organisms growing in chemostat culture at defined conditions, was estimated on the basis of the dilution rate, the total nitrogen content of cultures at steady state corrected for the amount of ammonium sulfate supplied with the feed medium (Klein *et al.*, 1993). Total nitrogen was determined by the micro Kjeldahl method according to Beloserski and Proskurjakow (1956). The potential nitrogenase activity was assayed by reduction with samples removed from the cultures. The assay system containing 9 mM of the electron donor, D,L-malate, was incubated under anaerobiosis and with saturating illumination (30 klx) (Klein *et al.*, 1993). The rate of cellular substrate consumption was determined on the basis of the concentration of D,L-malate as well as of cell protein at steady state and the dilution rate. Malate was determined with test combinations (Boehringer, Mannheim, FRG). β-Galactosidase activity of the *nifH-lacZ* fusion mutant was assayed with cell-free extracts obtained after passing washed cell suspensions through a French pressure cell (two passages at 97 megaPascal). 10 mM Tris(hydroxymethyl)aminomethane-hydrochloride pH 7.6 was used as buffer system. After removal of cell debris by centrifugation of homogenates at 27,000 x *g* for 5 min at 4°C, supernatants were assayed for β-galactosidase as described (Sambrook *et al.*, 1989). The activities were calculated on the basis of the absorption coefficient of *o*-nitrophenol at 420 nm, ε_{420} = 4500 $M^{-1}cm^{-1}$. *In situ* respiratory activities were calculated on the basis of the gas flow rate through the culture and the difference between oxygen concentrations at the gas inlet and the outlet of the chemostat (Bühler *et al.*, 1987).

Determination of nitrogenase polypeptides

Cell samples were frozen at the temperature of liquid nitrogen immediately upon removal from the culture. For sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) samples were thawed and washed by centrifugation at 4°C with deionized water. After addition of sample buffer (Laemmli, 1970) cells were solubilized by boiling for 3 min at 95°C. ADP-ribosylation was not lost during the handling of samples, as tested with samples subjected to ammonium shock or dark switch-off of nitrogenase. For ammonium shock, the ammonium sulfate concentration in the culture was suddenly increased from 0 at steady state to 3 mM. Separation of polypeptides by SDS-PAGE was performed as described (Laemmli, 1970) with 12.5 % (w/v) acrylamide and 0.6 % (w/v) N,N'-methylene bisacrylamide. Enzyme linked immunoassays were performed after Western blotting of nitrogenase polypeptides (Towbin *et al.*, 1979) and coupling with antibodies raised against Rc1 of *R. capsulatus* and component 2 from *R. rubrum* (Rr2). The polypeptide bands were visualized with a chemiluminescence assay (ECL detection system, Amersham) and subsequent exposure to X-ray film. For quantification, the bands were scanned and evaluated with a Biorad (Hercules, U.S.A.) GS 670 imaging densitometer. For each Western blot, linearity of the amount of nitrogenase polypeptides versus the staining intensity was confirmed using a standard nitrogenase preparation. The position of bands corresponding to the modified (ADP-ribosylated) and unmodified subunits of Rc2 were identified by comparison with results obtained with a standard nitrogenase preparation as well as with a diazotrophically grown phototrophic culture sample subjected to ammonium switch off of nitrogenase (Hallenbeck *et al.*, 1982).

Results

In situ nitrogen fixation

Previously it was reported that nitrogen-limited aerobic chemostat cultures of *R. capsulatus* fixed nitrogen only if exposed to oxygen concentrations below 2 % air saturation, while the potential activity of acetylene reduction by nitrogenase was detectable with cell samples grown at oxygen concentrations up to 30 % air saturation (Klein *et al.*, 1993). Since nitrogen fixation requires relatively high amounts of reducing equivalents and energy, it appeared possible that cells did not fix nitrogen at increased oxygen concentrations because of an insufficient supply of these factors. Alternatively, it might be presumed that oxygen directly inhibits nitrogenase. In the latter case, the question arises whether removal of oxygen by respiration might be capable of preventing this inhibition. In order

Table I. The effect of incandescent light on nitrogenase expression and function as well as on respiration in *Rhodobacter capsulatus* growing at steady state in aerobic chemostat culture. The organisms were supplied with 25 mM D,L-malate and 0.5 mM ammonium sulfate at a dilution rate of 0.05 h^{-1}. Ambient oxygen concentrations were adjusted with air to 1% and 5% air saturation.

Parameter	1% air saturation			5% air saturation	
	dark	3 klx	30 klx	dark	30 klx
Protein[a]	0.42±0.03	0.5±0.03	0.72±0.03	0.1±0.01	0.09±0.1
N_2 fixed[b]	2±0.3	2.5±0.2	3.9±0.2	n.d.[e]	n.d.[e]
Acetylene red.[c]	32±6	34±6	38±7	9±1	0.6±0.1
β-Gal.ase[c,d]	21±2	–	27±1.5	13±1	5±0.5
Respiration[c]	122±13	65±2	18±7	92±12	42±18

Values given as [a] mg per ml, [b] mM, [c] nmol per min per mg cell protein [d] *nifH* promotor activity determined by the β-galactosidase activity of a *nif-lacZ* fusion mutant of *Rhodobacter capsulatus*. [e] n.d. = not detectable.

to answer these questions, the cultures were grown under conditions, which lead to variations in cellular respiration and to increases in the supply of energy or reducing equivalents. Energy regeneration may be enhanced upon illumination of aerobic dark cultures. In addition, it is known that illumination may inhibit cellular respiration (Oelze and Weaver, 1971).

The data compiled in Tables I and II confirmed that chemostat cultures of *R. capsulatus* fixed nitrogen only when adapted to 1 % air saturation but not when growing at 2 or 5 % air saturation. However, the steady state level of biomass (cell protein) as well as of nitrogen fixed by nitrogenase almost doubled when chemostat cultures growing at 1 % air saturation in the dark were illuminated with 30 klx (Table I). Nevertheless, the specific rate of nitrogen fixation (Klein *et al.*, 1993) and the potential nitrogenase activity, assayed with culture samples by acetylene reduction under optimum conditions, remained largely unaffected when the culture was illuminated. On the other hand, if adapted to 5 % air saturation, the organisms fixed no nitrogen and the activity of acetylene reduction was severely inhibited upon illumination. As expected, the rate of oxygen consumption by the cultures adapted to both of the oxygen concentrations significantly decreased in the light.

Table II. Specific rates of D,L-malate consumption (q), whole cell respiration and nitrogen fixation of *Rhodobacter capsulatus* growing in chemostat cultures at ambient oxygen concentrations of 1 % and 2 % air saturation in the dark. The cultures were grown under steady state conditions supplied with 25 mM D,L-malate and 0.5 mM ammonium sulfate at different dilution rates D (h^{-1}) as indicated.

Air satn.	Dilution rate	q[a]	Respiration[b]	N_2 fixation[c]
1%	0.05	48	122±13	4.2±0.8
	0.08	80	172±20	6.6±1.0
	0.12	130	176±17	5.0
2%	0.05	59	100±12	n.d.[d]
	0.08	98	158±28	n.d.
	0.12	152	192±8	n.d.

The values are presented as nmol of [a] malate and [b] O_2 consumed as well as [c] of N_2 fixed per mg of cell protein and min. [d] n.d. = not detectable.

According to the theory of chemostat cultures, increasing the dilution rate increases the rate of substrate supply and thus, in the present case, the rate at which the electron donor is supplied. This experimental approach was used to answer the question of whether cells did not fix nitrogen at higher oxygen concentrations because of lack of electron donor. Chemostat cultures were adapted to grow at different dilution rates and either at 1% or 2 % air saturation. With both cultures, the rate of substrate consumption increased with increasing the dilution rate (Table II). Concomitantly, cellular respiration increased as well. But neither increased substrate consumption nor increased oxygen uptake allowed the occurrence of cellular nitrogen fixation in cultures adapted to 2 % air saturation.

Activity of the nifH promoter

In order to study the extent of the control of nitrogenase expression by oxygen at the level of the *nifH* promoter, a transcriptional *nifH-lacZ* fusion was constructed. The resulting strain R372 of

R. capsulatus, carrying the chromosomal *nifH-lacZ* fusion, was grown at different oxygen concentrations under essentially the same conditions as the wild-type. Samples from steady states were withdrawn from chemostats and prepared for β-galactosidase assays. The results shown in Fig. 1A reveal that the *nifH* promoter was active within a range of 1 % to about 40 % of air saturation of the medium. Increasing the oxygen concentration from 1 to 40 % air saturation resulted in a biphasic inhibitory kinetic of *nifH* expression with a rather steep decrease between 1 and 2 %, a plateau between 2 and 10 % followed by a second steep decrease between 10 and 20 %. The activity leveled off between 20 and 40% air saturation.

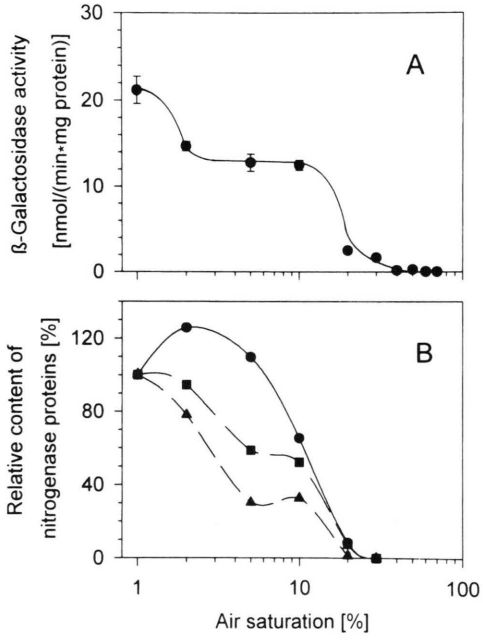

Fig. 1. Dependence of nitrogenase expression in *R. capsulatus* on the ambient oxygen concentration (100% air saturation corresponds to 230 μM dissolved oxygen). Chemostat cultures were grown aerobically in the dark at a dilution rate of $D = 0.05\ h^{-1}$. In order to limit the relative supply of bound nitrogen, the feed medium contained D,L-malate and ammonium sulfate at a C/N ratio of 100 (ratio of atoms).
A. β-Galactosidase activity of crude extracts of the *nifH-lacZ* fusion mutant of *R. capsulatus*.
B. Relative amounts of the α- (■) and β-polypeptides (▲) of Rc1 and the Rc2 (●) polypeptide of the nitrogenase complex of wild-type strain B10. The average values of densitometric evaluations of three independent chemostat experiments are depicted as percent of the respective concentrations of the three polypeptides determined at 1 % air saturation.

For control, we determined the β-galactosidase activity of anaerobic light cultures grown at 30 klx of incandescent light under otherwise identical conditions. Interestingly, the latter activity was 24 μmol/ min and mg of protein as compared to 21 μmol/ min and mg of protein characteristic of dark cultures adapted to 1 % air saturation. Accordingly, illumination of cultures growing diazotrophically at 1 % air saturation resulted in a slight, if any, increase in *nifH* promoter activity (Table I). Yet, at 5 % air saturation, illumination significantly decreased the activity of the *nifH* promotor (Table I).

Steady state levels of nitrogenase polypeptides

Since the activity of acetylene reduction by nitrogenase and the activity of the *nifH* promoter exhibited comparable sensitivities toward oxygen, it might be assumed that the occurrence of the α- and β-polypeptides of Rc1 as well as of the Rc2 polypeptide should follow the same pattern of sensitivity. In order to confirm this assumption, Western-blots of nitrogenase polypeptides obtained from aerobically grown cultures of the wild-type were quantitatively evaluated. Indeed, the results revealed that all of the three polypeptides were detectable below about 20 % air saturation of the medium (Fig. 1B). However, apart from this common feature, the three polypeptides showed significant differences in their respective cellular steady state levels. Obviously, the Rc2 polypeptide was the least sensitive toward oxygen; its relative content increased with increasing the oxygen concentration from 1 to about 2 % air saturation. Upon further increases of the oxygen concentration, the level of Rc2 steadily decreased and became undetectable at 30 % air saturation. By contrast, the levels of the α- and β-polypeptides of Rc1 showed biphasic dependencies on oxygen, where the relative levels of both decreased between 1 and 5 % air saturation, attained plateaus between 5 and 10 % and became undetectable when the oxygen concentration was raised to about 20 % air saturation. Overall, the β-polypeptide was more sensitive than the α-polypeptide. The differences in steady state levels of Rc1 and Rc2 polypeptides observed at different oxygen concentrations were rather unexpected. This is particularly true for the α and β polypeptides of

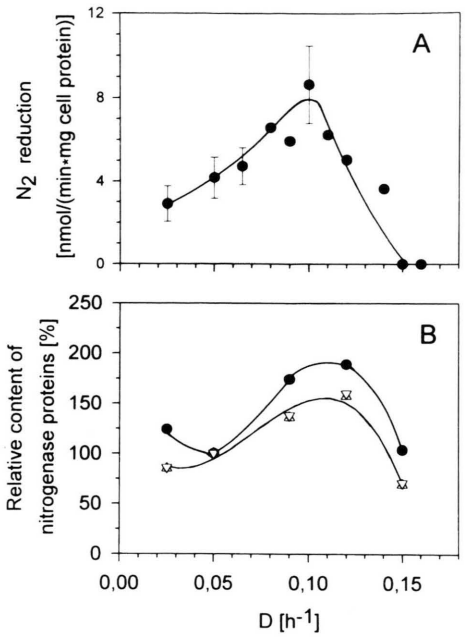

Fig. 2. Dependence of the expression and function of nitrogenase of *Rhodobacter capsulatus* on the dilution rate. The organisms were grown in dark chemostat culture at 1 % air saturation as described in Fig. 1.
A. *In situ* nitrogen fixation of cells (modified after Klein *et al.*, 1993).
B. Relative amounts of the α- (△) and β- (▽) polypeptides of Rc1 and the Rc2 polypeptide (●). The average values of densitometric evaluations of three independent experiments are expressed as percent of the value at D = 0.05 h^{-1}.

Rc1, which are assumed to form a tight Rc1 complex. Experimental error can be largely excluded because each data point of Fig. 1B is based on results obtained with samples from three independent chemostat cultures. Moreover, changes in the dilution rate at a constant oxygen concentration of 1 % air saturation are known to lead to considerable changes in nitrogenase activity (Klein *et al.*, 1993) as well as in the cellular levels of the three nitrogenase polypeptides (Fig. 2). In the latter case, the stoichiometric relationship particularly of the α and β polypeptides of Rc1 remained constant.

Inactive nitrogenase with unmodified Rc2

ADP-ribosylated and unmodified Rc2 exhibit molecular masses of 38 and 33.5 kDa, respectively (Jouanneau *et al.*, 1983). In the present investigation, quantitative determinations of Rc2 could be restricted to the unmodified form of the polypeptide because modified Rc2 was not detectable with any of the samples from aerobic cultures of the wild-type of *R. capsulatus* (Fig. 3). In order to confirm that modification of Rc2 was not required for maintaining significant cellular steady state levels

Fig. 3. Enzyme-linked immunosorbent assay of the Rc2 polypeptides of nitrogenase of the wild-type (slots 1–4) and the DraT/DraG negative mutant W107I (slots 6–9) of *R. capsulatus* grown as described in Fig. 1. The modified (ADP-ribosylated) and unmodified polypeptides exhibit molecular masses of 38 and 33.5 kDa, respectively (Jouanneau *et al.*, 1983) Slots 1–3 and 7–9: samples taken from cultures grown at 1 % (slots 1 and 7), 5 % (slots 2 and 8) and 10 % (slots 3 and 9) air saturation. Each slot was loaded with 32 μg of cell protein. Slot 5: 0.03 μg of isolated Rc2. Slots 4 and 6: reference samples taken from phototrophic batch cultures after a sudden increase in the ammonium concentration, 8 μg of cell protein were loaded on top of each slot.

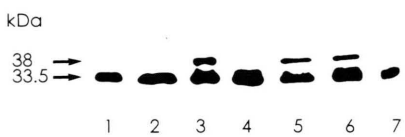

Fig. 4. The effects of oxygen stress conditions or of an ammonium shock on the banding patterns of Rc2 of the nitrogenase complex of aerobically grown *R. capsulatus* strain B10. The organisms were grown at 1 and 5% air saturation as in Fig. 1. As soon as steady states had been confirmed, the different shock conditions were set and samples were analyzed as in Fig. 1 for the occurrence of the modified (ADP-ribosylated) form (38 kDa) of Rc2. A nitrogen fixing chemostat culture was subjected to a sudden increase in the oxygen concentration from 1 to 50 % air saturation. A sample taken after 60 minutes of stress is shown in slot 1. Slot 2: Control sample derived from a culture growing under steady state conditions at 1 % air saturation. Slots 3 and 5: Ammonium shock. The ammonium sulfate concentration in the culture was suddenly increased from 0 to 3 mM. After 15 minutes of shock, samples were taken from cultures adapted to 1% (slot 3) or 5 % air saturation (slot 5). Slot 4: Control sample derived from a culture growing under steady state conditions at 5 % air saturation. Slot 6: reference sample taken from phototrophic batch cultures after a sudden increase in the ammonium concentration. Slots 1–6: 15 μg of cell protein were loaded on each slot. Slot 7: reference sample of 0.02 μg of isolated unmodified Rc2.

of potentially active nitrogenase under aerobiosis, strain W107I of *R. capsulatus* was included in the present studies. As expected, the modified Rc2 polypeptide of 38 kDa was not detectable in the mutant (Fig. 3). Nevertheless, with respect to *in situ* nitrogen fixation and the acetylene reduction activity of nitrogenase, the mutant exhibited the same sensitivity to oxygen as the wild-type. These results led to the question, if under aerobiosis nitrogenase is still susceptible to modification. This question was answered by exposing wild-type cells of *R. capsulatus* adapted to 1 and 5 % air saturation to ammonium shock conditions, which unequivocally led to modification of Rc2 (Fig. 4). Interestingly, modification was possible irrespective of whether the cells fixed nitrogen or not.

Oxygen stress conditions

Slow ADP-ribosylation was reported to occur in *R. rubrum* under oxygen stress (Kanemoto and Ludden, 1984). The experiments detailed so far were performed with cultures adapted to different oxygen concentrations. Thus, it seemed possible that under steady state conditions the correct conditions for modifying Rc2 were not met. Consequently, aerobic dark chemostat cultures adapted to 1 % air saturation were subjected to an oxygen stress of 50 % air saturation for 60 min. At this high oxygen concentration nitrogenase synthesis is completely inhibited (Fig. 1B). Analyses of Western blots of the three polypeptides of the nitrogenase complex revealed a parallel decrease in their levels by 24–22 %, about 5 % of which resulted from dilution in the course of 60 min of stress at a dilution rate of 0.05 h^{-1} (Table III). However, even under these conditions, the presence of the modified form of Rc2 could not be detected (Fig. 4). In the course of 60 min of oxygen stress the activity of acetylene reduction decreased by 40 % (Table III).

Table III. The effect of oxygen stress conditions on the relative levels of the α and β polypeptides of component 1 (Rc1) and of component 2 (Rc2) as well as on the acetylene reduction activity of nitrogenase in *Rhodobacter capsulatus* wild-type strain B10. Dark chemostat cultures fixing dinitrogen at 1 % air saturation and a dilution rate of 0.05 h^{-1} were subjected to an oxygen stress of 50 % air saturation for 60 minutes. The results represent averages of three independent stress experiments.

Nitrogenase parameter	0[a] min	30[a] min	50[a] min
Relative[b] amount of			
Rc1α	100	93	78
Rc1β	100	91	76
Rc2	100	96	76
Relative[b] acetylene reduction activity	100	–	61

[a] min of stress, *i. e.* min exposure from 1 to 50% air saturation; [b] given as percent of the respective values before oxygen stress (time: 0 min).

Discussion

Upon illumination of chemostat cultures adapted to 1 % air saturation the steady state levels of both biomass (protein) and nitrogen fixed significantly increased. This indicates that energy regeneration by aerobic cells of *R. capsulatus* can be enhanced under phototrophic conditions. But at a slightly higher oxygen concentration of 5 % air saturation, illumination did not activate nitrogen fixation. This largely excludes that at the higher oxygen concentrations nitrogen fixation was lacking because of insufficient energy supply. This conclusion is supported by results showing that nitrogen fixation by dark cultures adapted to 2 % air saturation could not be activated by increasing the dilution rate and, thus, the rate of energy regeneration coupled to increased respiration. Moreover, the latter results indicate that increasing the supply of electron donor did not either activate nitrogen fixation. This, however, does not exclude the possibility that oxygen interfers with the electron transfer to nitrogenase. Finally, the experiments discussed so far reveal that, under the present conditions, oxygen consumption by whole cells of *R. capsulatus* was not significantly involved in the controls of either nitrogen fixation, the *nifH* promoter activity or the potential activity of nitrogenase. This conclusion is based on the following results: Illumination of cultures adapted to 1 % air saturation was of no significant effect with respect to the activities of the *nifH* promotor or acetylene reduction. But, although light decreased the respiratory activity by about 90 %, the total amount of nitrogen fixed by nitrogenase increased upon illumination. Moreover, after increasing the oxygen concentration from 1 to 2 % air saturation, the activity of nitro-

gen fixation could not be preserved, even if this increase was accompanied by a doubling of the respiratory activity. Therefore, it appears very likely that the presently observed effects of oxygen on nitrogenase were direct rather than indirect *via* changes in oxygen consumption, energy regeneration or the supply of reducing equivalents.

According to the present data, various levels involved in the expression of nitrogenase and the occurrence of nitrogen fixation exhibited considerable differences with respect to their sensitivities to oxygen. By far the highest sensitivity was identified for nitrogen fixation, while the activity of the *nifH* promoter was the least sensitive. The biphasic inhibitory kinetics of the activity of the *nifH* promoter suggests that at least two steps with different sensitivities to oxygen may be involved in the activation of this promoter. Since NifA is required for transcription of *nif* genes and since *R. capsulatus* was shown to possess two copies of *nifA*, it may be speculated that either transcription of both copies of *nifA* or the resulting polypeptides NifAI and NIFAII, which differ in their N-terminal amino acid sequences, exhibit different sensitivities to oxygen (Foster-Hartnett and Kranz, 1992; Preker *et al.*, 1992; Hübner *et al.*, 1993). As, yet, however, detailed investigations on the oxygen sensitivity of *nifA* transcription or the stability of NifA in *R. capsulatus* are lacking.

A biphasic type of inhibitory kinetic could be identified not only at the levels of acetylene reduction (Klein *et al.*, 1993) and the *nifH* promoter activity but also at the level of α- and β-polypeptides of Rc1. However, the relative steady state contents of the two polypeptides were differently affected by oxygen, where the β-subunit exhibited the higher sensitivity. In light of the results discussed so far, it is noteworthy that steady state levels of Rc2 significantly deviated from this behaviour.

The different steady state levels of Rc1 and Rc2 may be assumed to result from different stabilities of the subunits in the presence of oxygen. However, the present investigation showed that different *in vivo* stabilities of Rc1 and Rc2 against oxygen can be largely excluded because oxygen stress conditions affected the cellular levels of all of the three polypeptides only slightly and equally. This and the fact that *nifHDK* genes are transcribed from a common promoter suggest the involvement of oxygen sensitive posttranscriptional sites in the control of nitrogenase. In light of results indicating that different ratios of Rc1 to Rc2 may arise from different ratios of *nifDK* mRNA to *nifH* mRNA (Willison *et al.*, 1993) it appears possible that oxygen controls the levels of the respective mRNA's.

According to the present study, inactivation of nitrogenase under aerobic growth conditions or oxygen stress was not accompanied by the formation of the modified form of Rc2. Since the present investigations were performed with cultures grown in the dark, it may be concluded that, in *R. capsulatus*, the absence of light is not sufficient to stimulate ADP-ribosylation, which was previously observed with phototrophic cells of *R. rubrum* and *R. capsulatus* if the light source was suddenly removed (Pierrard *et al.*, 1993). Nevertheless, if under aerobic dark conditions the ammonium concentration was suddenly increased, Rc2 was susceptible to modification irrespective of whether the cultures fixed nitrogen or not. From this, it can be concluded that modification of Rc2 does not require the enzyme in its active state.

Acknowledgements

The authors thank P. Ludden for helpful discussions and for antisera against Rr2, K. Schneider for antisera against Rc1, Y. Jouanneau for *R. capsulatus* nitrogenase proteins and U. Schramm for providing unpublished plasmids.

Beloserski A. N., and Proskurjakow L. (1956), Praktikum der Biochemie der Pflanzen. Deutscher Verlag der Wissenschaft. Berlin.

Beynon J. L., Williams M. K. and Cannon F. C. (1988), Expression and functional analysis of the *Rhizobium meliloti nifA* gene. EMBO J. **7,** 7–14.

Bühler T., Sann R., Monter U., Dingler C. and Oelze J. (1987), Control of respiration and growth yield in ammonium-assimilating cultures of *Azotobacter vinelandii*. Arch. Microbiol. **148,** 242–246.

Burris R. H. (1991), Nitrogenase. J. Biol. Chem. **266,** 9339–9342.

Fischer H. M. and Hennecke H. (1987), Direct response of *Bradyrhizobium japonicum nifA*-mediated *nif* gene regulation to cellular oxygen status. Mol. Gen. Genet. **209,** 621-626.

Foster-Hartnett D. and Kranz R. G. (1992), Analysis of the promoters and upstream sequences of *nifA1* and *nifA2* in *Rhodobacter capsulatus*: activation requires *ntrC* but not *rpoN*. Mol. Microbiol. **6,** 1049–1060.

Gallon, J. R. (1992), Reconciling the incompatible: N_2 fixation and O_2. New Phytol. **122,** 571–609.

Gussin G. N., Ronson C. W. and Ausubel F. M. (1986), Regulation of nitrogen fixation genes. Annu. Rev. Genet. **20,** 567–591.

Hallenbeck P. C., Meyer C. M. and Vignais P. M. (1982), Nitrogenase from the photosynthetic bacterium *Rhodopseudomonas capsulata*: purification and molecular properties. J. Bacteriol. **149,** 708–717.

Hübner P., Masepohl B., Klipp W. and Bickle T. A. (1993), *Nif* gene expression studies in *Rhodobacter capsulatus*: *ntrC*-independent repression by high ammonium concentrations. Mol. Microbiol. **10,** 123–132.

Jouannneau Y., Meyer C. M. and Vignais P. M. (1983), Regulation of nitrogenase activity through iron protein interconversion into an active and an inactive form in *Rhodobacter capsulatus*. Biochim. Biophys. Acta **749,** 318–328.

Kanemoto R. H. and Ludden P. W. (1984), Effect of ammonia, darkness and phenazine methosulfate on whole-cell nitrogenase activity and Fe protein modification in *Rhodospirillum rubrum*. J. Bacteriol. **158,** 713–720.

Klein G., Jahn A., Dörffler M. and Oelze J. (1993), Activity and expression of nitrogenase in *Rhodobacter capsulatus* under aerobiosis in the dark. Arch. Microbiol. **159,** 233–236.

Kranz R. G. and Foster-Hartnett D. (1990), Transcriptional regulatory cascade of nitrogen fixing genes in anoxygenic photosynthetic bacteria: oxygen- and nitrogen responsive factors. Mol. Microbiol. **4,** 1793–1800.

Kranz R. G. and Haselkorn R. (1986), Anaerobic regulation of nitrogen fixation genes in *Rhodopseudomonas capsulata*. Proc. Natl. Acad. Sci. USA **83,** 6805–6809.

Labes M., Pühler A., Simon R. (1990), A new family of RSF1010-derived expression and *lac*-fusion broad-host-range vectors for Gram-negative bacteria. Gene **89,** 7–46.

Laemmli U. K. (1970), Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227,** 680–685.

Ludden P. W. and Roberts G. P. (1995), The biochemistry and genetics of nitrogen fixation by photosynthetic bacteria. In: Anoxygenic Photosynthetic Bacteria (Blankenship R. E., Madigan M. T. and Bauer C. E., eds.), Kluwer Academic Publishers, Dordrecht, Boston, London, pp. 929–947.

Masepohl B., Klipp W. and Pühler A. (1988), Genetic characterization and sequence analysis of the duplicate *nifA/nifB* gene region of *Rhodobacter capsulatus*. Mol. Gen. Genet. **212,** 27–37.

Masepohl B., Krey R. and Klipp W. (1993), The *draTG* gene region of *Rhodobacter capsulatus* is required for post-translational regulation of both the molybdenum and the alternative nitrogenase. J. Gen. Microbiol. **139,** 2667–2675.

Oelze J., and Weaver P. (1971), The adjustment of photosynthetically grown cells of *Rhodospirillum rubrum* to aerobic light conditions. Arch. Microbiol. **79,** 108–121.

Pierrard J., Ludden P. W. and Roberts G. P. (1993), Posttranscriptional regulation of nitrogenase in *Rhodobacter capsulatus*: existence of two independent regulatory effects of ammonium. J. Bacteriol. **175,** 1358–1366.

Pierrard J., Willison J. C., Vignais P. M., Gaspar J. L., Ludden P. W. and Roberts G. P. (1993), Site-directed mutagenesis of the target arginine for ADP-ribosylation of nitrogenase component II in *Rhodobacter capsulatus*. Biochim. Biophys. Res. Commun. **192,** 1223–1229.

Post E., Golecki J. R. and Oelze J. (1982), Morphological and ultrastructural variations in *Azotobacter vinelandii* growing in oxygen-controlled continuous culture. Arch. Microbiol. **133,** 75–82.

Preker P., Hübner P., Schmehl M., Klipp W. and Bickle T. A. (1992), Mapping and characterization of the promoter elements of the regulatory *nif* genes *rpoN*, *nifA1*, and *nifA2* in *Rhodobacter capsulatus*. Mol. Microbiol. **6,** 1035–1047.

Robson R. L. and Postgate J. R. (1980), Oxygen and hydrogen in nitrogen fixation. Annu. Rev. Microbiol. **34,** 183–207.

Sambrook J., Fritsch E. F. and Maniatis T. (1989), Molecular cloning. A laboratory manual. 2. Edition. Cold Spring Harbor Laboratory Press, New York.

Siefert E. and Pfennig N. (1980), Diazotrophic growth of *Rhodopseudomonas acidophila* and *Rhodopseudomonas capsulata* under microaerobic conditions in the dark. Arch. Microbiol. **125,** 73–77.

Towbin H., Staehelin T. and Gordon J. (1979), Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. USA **76,** 4350–4354.

Weaver P. F., Wall J. D. and Gest H. (1975), Characterization of *Rhodopseudomonas capsulata*. Arch. Microbiol. **105,** 207–216.

Willison J. C., Pierrard J. and Hübner P. (1993), Sequence and transcription analysis of the nitrogenase structural gene operon (*nifHDK*) of *Rhodobacter capsulatus*: evidence for intramolecular processing of *nifHDK* mRNA. Gene **133,** 39–46.