Biochimica et Biophysica Acta 808 (1985) 149–155
Elsevier

BBA 41772

Stimulation by light of nitrogenase synthesis in cells of *Rhodopseudomonas capsulata* growing in N-limited continuous cultures

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(Received December 27th, 1984)

Key words: Nitrogenase; Enzyme induction; (*Rps. capsulata*)

In cells of *Rhodopseudomonas capsulata* growing in nitrogen-limited continuous culture the nitrogenase-specific activity was found to be closely dependent on the light intensity. As light intensity, measured with a photodiode immersed in the culture, was varied stepwise from 1000 to 7000 lux, the nitrogenase activity, measured at steady state, increased gradually up to 5-fold. Shifting light intensity from 1200 to 7000 lux resulted in a sharp rise in nitrogenase activity which doubled within the first two hours. The determination by immunoassays of the intracellular levels of each nitrogenase component revealed that the light-dependent stimulation of nitrogenase activity was correlated with the accumulation of the nitrogenase enzyme inside the cells. Under high illumination, nitrogenase represented up to 40% of the cytoplasmic proteins. The specific activities of each component in intact cells, calculated on the basis of their relative concentration in the cells and on in vivo nitrogenase assays, appeared roughly constant and hardly affected by changes of light intensity. The specific activity of the Fe protein was about 7-fold higher in intact cells than in the purified state. The ratio of the two nitrogenase components remained fairly constant and close to one, irrespective of the light intensity to which cells were exposed. These results demonstrate that in nitrogen-limited grown cells of *Rps. capsulata* light brings about an induction or a derepression of nitrogenase synthesis the extent of which is dependent on light intensity.

Introduction

The purple nonsulfur bacterium *Rhodopseudomonas capsulata* has a great capacity to produce hydrogen gas in the light, while degrading small organic molecules [1,2]. This ability results from the activity of nitrogenase (reduced ferredoxin : dinitrogen oxidoreductase (ATP-hydrolyzing); EC 1.18.2.1.) which catalyzes the reduction of protons by an ATP-dependent reaction. In phototrophic bacteria, nitrogenase activity is regulated both at the transcriptional and the enzymatic levels in response to several environmental factors including tension, concentration of nitrogen source and light intensity (see Ref. 3 for a review). Excess of oxygen or ammonia produces severe repression of nitrogenase in *Rps. capsulata* [4]. In addition, ammonia brings about a short-term, total and reversible inhibition of nitrogenase, the so-called 'switch-off effect' [5,6]. The mechanism of this regulation involves a reversible inactivation of the iron protein by attachment to or release from one of its subunits of a specific modifying group [7–9]. An activating enzyme [10] catalyzes the removal of the

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Abbreviations: Rc1 and Rc2, nitrogenase components I and II from *Rhodopseudomonas capsulata*, respectively; Av2, nitrogenase component II from *Azobacter vinelandii*

modifying group by a reaction requiring ATP and divalent cations.

From the studies of Hillmer and Gest [1,2], light intensity appeared to stimulate greatly the H_2-evolving capacity of batch cultures of *Rps. capsulata*. In nitrogen-limited continuous cultures, we previously observed that H_2 evolution as well as specific acetylene reduction could be enhanced more than 5-fold upon exposure to increasing illumination [11,12]. We suggested that the stimulation of nitrogenase activity was due to a light-induced de novo synthesis of the enzyme. However, light could also produce activation of nitrogenase through Fe protein demodification [9,13].

To discriminate between mere activation and de novo nitrogenase synthesis specific antibodies were raised against each nitrogenase component (Fe protein and MoFe protein) and used to estimate the concentration of nitrogenase proteins in intact cells. They bring evidence that the light-dependent stimulation of nitrogenase activity results from the intracellular accumulation of nitrogenase components. Quantitation of nitrogenase Fe protein and MoFe protein by rocket immunoelectrophoresis indicated that each of the two components increased with light intensity to the same extent so that the ratio of Fe protein to a MoFe protein remained constant and close to 1 indicating that the synthesis of these proteins are coregulated.

Materials and Methods

Culture conditions

Rps. capsulata strain B10 (obtained from the Photosynthetic Bacteria Unit, Indiana University, Bloomington, IN, U.S.A.) was grown in a Bioflo C30 fermenter (New Brunswick Scientific Co. Inc., U.S.A.) of 0.45L working capacity. Growth conditions were as described [12] and glutamate concentration in the inflowing medium was 3 mM.

Illumination and light intensity measurements

Illumination of the culture was provided by two 100 W incandescent lamps placed in diametrically opposite positions at identical distances from the culture vessel. Light intensity was measured with a small planar photodiode (Diode Hamamatsu S1133-14) which was calibrated to a luxmeter. The photodiode, introduced inside a glass tube of 1 cm in diameter was placed in the axis of the culture vessel at half the depth of the culture. From the two maximal values read in the direction of each lamp, an average light intensity (I_a) was calculated. The light distribution inside the culture along the axis of the lamps was first determined. Experimental data could be fitted with a good approximation to an exponential: $f(I) = I_0\, e^{-dr}$, where d is the distance between the vessel wall and the photodiode; r is proportional to the bacterial cell density. Using this formula, the incident light intensity at the vessel surface in the axis of the lamps (I_0) could be calculated from the measurements taken in the center of the vessel. The intensity of incident light reaching the surface of the culture vessel was also averaged from measurements with the photodiode placed against the inner surface of the vessel and facing outside. From this value of incident irradiance, the average light intensity I_a through the culture was deduced by integration of the light distribution function along a radius of the culture vessel.

Nitrogenase assay

Nitrogenase activity was measured in vivo by acetylene reduction and H_2 evolution. The acetylene reduction assays were performed on 1 ml samples of culture as already described [9]. The rate of H_2 evolution from the culture was determined as in Ref. 12.

Concentration of nitrogenase components

Nitrogenase concentration in bacterial cells was determined by immunoelectrophoresis using specific antibodies raised against each pure nitrogenase component. Purification of nitrogenase components and preparation of antibodies were carried out according to published procedures [9]. Culture samples (3 to 10 ml) were centrifuged (10 min, 20000 × g), concentrated 20–40 fold in 0.125 M Tris-HCl (pH 8.0) and then disrupted by sonication for 1 min with a microsonicator, model W10 (Heat systems-Ultrasonics, Inc. Plainview, NY, U.S.A.). The crude homogenates were centrifuged (5 min, 90000 × g) in a Beckman Airfuge centrifuge to remove membrane fragments. Aliquot fractions (5 or 10 µl) were then analyzed by electrophoresis on agarose plates according to Plumley and Schmidt [14] except that polyethyleneglycol

was omitted. Standards of known amounts of pure MoFe protein (Rc1) and Fe protein (Rc2) were electrophoresed on the same plate. The plates were prepared by mixing, at 55°C, 10 ml of melted agarose with 50 µl of AntiRc1 or 150 µl of AntiRc2 rabbit serum and pouring it on plastic sheets (LKB, Bromma, Sweden) (84 × 94 mm). Electrophoresis was carried out at 5 V/cm for 4 h. The plates were then washed in 0.1 M NaCl, dried and stained with Coomassie blue. Quantitation of Rc1 and Rc2 in the samples was made by measuring the area of the immunoprecipitation rocket and comparing it to that of standards. Concentration refers to the protein content of each sample determined by the method of Lowry et al. [15].

Other determinations

Bacterial density was estimated by reading the absorbancy of the cell suspensions at 660 nm (A_{660}). The cell dry weight was deduced from the following equation: $A_{660} = 2 \times$ cell dry wt. (mg/ml). The protein content of cell extracts and of the purified nitrogenase samples was determined according to Lowry et al. [15].

Results

Effect of light intensity on the nitrogenase activity of Rps. capsulata grown in nitrogen-limited continuous culture

Rps capsulata was grown in a continuous culture with glutamate as limiting nitrogen source. The culture was exposed to various light intensities which were accurately measured with a calibrated photodiode immersed in the center of the culture. From the value given by the photodiode, an average light intensity was calculated taking into account the average absorption of the culture and the geometry of the culture vessel (see Methods). The values reported in the present study represent the average light intensity actually reaching the bacteria (and not the incident light intensity as given in earlier publications, e.g. in Ref. 12). Low irradiance, limiting for growth, was not used in order to be sure that bacteria were always under nitrogen deficiency. Under these conditions, at any light intensity, bacterial density reached the same steady-state value (0.45 mg dry wt./ml), since it depended only on the glutamate concentration in the fresh nutrient medium. Nitrogenase activity was determined either directly by the rate of H_2 evolved from the culture or by acetylene reduction assayed on culture samples under saturating illumination conditions.

As shown in Fig. 1, nitrogenase activity dramatically increased as a function of light intensity. Curves of H_2 production and acetylene reduction both exhibit a similar sigmoidal shape showing a sharp increase of activity in the range of 2000 to 4000 lx. Nitrogenase activity was 5-fold higher at 7000 lx than at 1500 lx. Since the acetylene reduction assay was performed under saturating illumination (approx. 10000 lx), the catalytic activity of nitrogenase determined in this assay should not be limited by provision of ATP or reductant. These results suggested that the variations of nitrogenase activity reflected differences in the amount of enzyme synthesized. To test this hypothesis, the amounts of nitrogenase components present in cell extracts were quantified by electroimmuno assays.

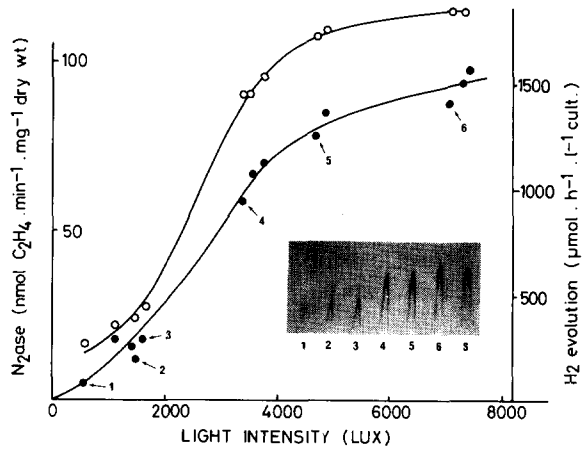

Fig. 1. Effect of light intensity on H_2 production and specific nitrogenase activity of *Rps. capsulata* cells grown in *N*-limited continuous culture. The dilution rate was set at 0.18 h^{-1}. Steady-state bacterial density reached 0.45 ± 0.05 mg dry weight/ml. H_2 evolution (○) was recorded directly at the fermenter outlet, while acetylene reduction (●) was assayed on 1 ml samples under standard illumination conditions (ca 10000 lx). Inset: rocket assay of nitrogenase component I (Rc1). Bacterial samples were taken as indicated and cell extracts were prepared. Aliquots (5 µl) were analyzed by electrophoresis on an agarose plate containing 5 µl/ml of antibodies antiRc1. Standard (S) contained 1.4 µg of pure Rc1.

TABLE I

LIGHT STIMULATION OF NITROGENASE SYNTHESIS IN *RHODOPSEUDOMONAS CAPSULATA*. LEVELS AND SPECIFIC ACTIVITIES OF EACH COMPONENT

Lux	In vivo N_2ase activity [a]	Relative proportion of cytoplasmic proteins		Calculated specific activity [b] of		Rc2/Rc1 ratio (mol/mol)
		Rc1	Rc2	Rc1	Rc2	
		(%)	(%)			
540	10	4.0	0.83	1077	5190	0.71
1400	13.5	4.4	1.24	1077	5560	0.97
1600	18	6.1	1.72	1272	4510	0.97
3400	59	20.5	4.32	1240	5890	0.72
4700	81.5	25.4	6.64	13320	5517	0.90
7200	91	26.6	7.11	1425	5700	0.92

[a] Measured in nmol $C_2H_4 \cdot min^{-1} \cdot mg^{-1}$ cell dry wt.
[b] Measured in nmol $C_2H_4 \cdot min^{-1} \cdot mg^{-1}$ protein (the method of calculation is indicated in the text).

Effect of light intensity on the synthesis of nitrogenase components in cells of Rps. capsulata growing in a continuous culture

In the experiment described in Fig. 1, culture samples were collected at the times indicated and analyzed for the amount of MoFe protein (Rc1) and Fe protein (Rc2) using specific antibodies raised against each pure protein. Bacteria in the samples were concentrated and disrupted by sonication. Cell debris and membrane chromatophores were removed by centrifugation at $90000 \times g$ for 5 min, and the cytoplasmic proteins of the supernatant were electrophoresed on agarose plates containing antibodies against Rc1 or Rc2. 'Rocket' patterns were obtained the size of which increased as a function of light intensity indicating that the synthesis of nitrogenase components is stimulated by light.

The amount of each component present in bacteria was estimated by comparison of the rocket areas with standards consisting of known amounts of pure Rc1 or Rc2 electrophoresed at the same time. As indicated in Fig. 2, the proportion of Rc1 and Rc2 in the cytoplasm appeared linearly related to the nitrogenase specific activity of whole cells. In other words, the increase of nitrogenase specific activity induced by light reflects the accumulation of nitrogenase components. Under high illumination, the cytoplasm contained up to nearly 30% of component I (Rc1) and up to 7% of component II (Rc2).

Assuming molecular weights of 230000 and 67000 for Rc1 and Rc2 respectively [16], one can calculate a molar ratio Rc2/Rc1 of 0.78 from the slopes of the curves presented in Fig. 2. The fact that the ratio is constant and close to 1 as light intensity varied suggests that the 2 nitrogenase components are cosynthesized. This led us to investigate the kinetics of nitrogenase synthesis upon induction by light.

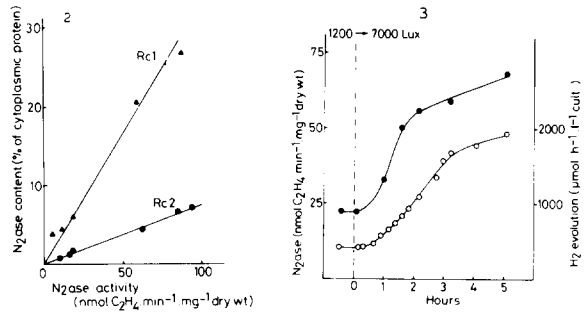

Fig. 2. Relationship between nitrogenase specific activity and the amount of Rc1 and Rc2 in the cytoplasm. Rc1 and Rc2 were quantified by immunoelectrophoresis as indicated in the text.

Fig. 3. Effect of a light shift up on nitrogenase activity of *Rps. capsulata* cells grown in *N*-limited continuous culture. The dilution rate was set at 0.091 h^{-1}. At zero time light intensity was shifted from 1200 up to 7000 lx. Steady-state bacterial density was 0.45 ± 0.05 mg dry wt./ml. H_2 evolution (○) and acetylene reduction (●) were determined as indicated in Fig. 1.

Stimulation of nitrogenase synthesis by light

Rps. capsulata grown in a glutamate-limited continuous culture under low light intensity (1200 lx) was suddenly exposed to a high light intensity (7000 lx). The nitrogenase specific activity and H_2 production from the culture were measured during the time following the light shift-up (Fig. 3). Both determinations indicated that the enhancement of illumination brought about a quick increase of nitrogenase activity. Within 2 h acetylene reduction activity increased about 2.5-fold. The curve of H_2 evolution is comparable to that of acetylene reduction activity. If the low H_2 evolution observed at 1200 lx resulted merely from a limitation of the provision of ATP or reductant to nitrogenase, then H_2 evolution would increase immediately upon exposure to a much higher intensity (7000 lx). Instead, the curve of H_2 evolution exhibited a lag of about 30 min after the light shift-up which could correspond to the time required to initiate nitrogenase synthesis. Therefore, the increase of nitrogenase activity could result from a light-induced specific stimulation of nitrogenase synthesis. We determined the intracellular amount of the enzyme components on bacterial samples taken at time intervals after the light shift-up. Table II shows that the increase of nitrogenase specific activity was closely correlated to the accumulation of both enzyme components in the cytoplasm of the bacteria. The calculated molar ratio Rc2/Rc1 was roughly constant and close to one indicating that light induced (or derepressed) the cosynthesis of both nitrogenase components.

Specific activity of Rc1 and Rc2

From the nitrogenase specific activity determined in vivo and the relative content of each enzyme component in the cytoplasm, one can calculate the specific activity of each component. We assumed that the total cell proteins and the cell dry weight were in the ratio 1 : 2.5 [17] and determined by Lowry assays [15] that the cytoplasm contained 58% of the cell proteins. The measured activities (Tables I and II) showed some variation due mainly to the lack of accuracy in the determination of the amount of Rc1 and Rc2 by immunoelectrophoresis, especially under low illumination. However, the results obtained from the separate experiments (Tables I and II) are in good agreement and show that the two nitrogenase components exhibited roughly constant in vivo activities under a wide range of light intensities. Activities were, however, slightly higher under high illumination.

In the purified state nitrogenase components of *Rps. capsulata* exhibit specific activities in the range of 1500–1700 for Rc1 and 600–800 for Rc2 [16]. Rc1 displayed in vivo activities comparable to, but slightly lower than that of the isolated form indicating that it was not fully active in intact cells. In contrast, the in vivo specific activities of Rc2 were 6–7-fold higher than that of the pure protein (Tables I and II). Similarly, the specific activity of Av2 in *Azotobacter vinelandii* was found to be much higher in intact cells than in the purified state [18]. These results indicate that, aside from a possible denaturation of Fe protein during purification, intact cells either have an electron-donat-

TABLE II

TIME-COURSE OF THE ACCUMULATION OF NITROGENASE COMPONENTS AFTER A LIGHT SHIFT-UP

Time after light shift-up (1200–700 lx)	In vivo nitrogenase activity [a]	Relative proportion of cytoplasmic proteins		Calculated specific activty [b] of		Rc2/Rc1 ratio (mol/mol)
		Rcl (%)	Rc2 (%)	Rc1	Rc2	
0 05	22.5	7.8	2.2	1245	4413	0.96
1 00	33.2	12.5	3.85	1145	3720	1.06
1.36	50.7	18.4	4.2	1150	5200	0.76
2.10	56.0	17.8	4.2	1400	5747	0.83
5 05	68.8	20.2	5.05	1467	5870	0.86

[a] Measured in nmol $C_2H_4 \cdot min^{-1} \cdot mg^{-1}$ cell dry wt.

[b] Measured in nmol $C_2H_4 \cdot min^{-1} \cdot mg^{-1}$ protein (the method of calculation is indicated in the text).

ing system to Fe protein more efficient than the system used in vitro or offer better conditions for the complexation of the two components than the in vitro system.

Discussion

Under *N*-limitation, the nitrogenase activity of *Rps. capsulata* was dramatically stimulated by light. By using specific antibodies against each nitrogenase components, we could estimate the intracellular concentration of nitrogenase proteins and demonstrate that the light-induced increase of nitrogenase activity was correlated with an intracellular accumulation of the nitrogenase enzyme. Under high illumination nitrogenase represented up to 40% of the total cytoplasmic proteins (Fig. 2). Such an overproduction of nitrogenase proteins has been observed previously in *Rhodopseudomonas palustris* maintained under conditions of *N*-deprivation [19]. The authors suggested that the extent of nitrogenase derepression was correlated with the degree of nitrogen deficiency.

In our culture conditions we made sure that, even under low illumination, light intensity was never limiting for growth, as judged by the stability of the steady state bacterial density. Then, according to the laws of continuous culture [20], the concentration of the limiting nitrogen source, was constant at a constant dilution rate. Therefore, at any light intensity, the bacteria were equally deprived of nitrogen nutrient and the observed variation of nitrogenase levels in the cells could not arise, in our case, from variations of the degree of *N*-deficiency. Hence, the overproduction of nitrogenase actually resulted from an intrinsic effect of light on nitrogenase synthesis.

The accumulation of nitrogenase may arise from either an enhanced rate of synthesis or a reduced rate of degradation. The half-life of nitrogenase in *N*-limited cells was found to be longer than 50 h [19]. Therefore the degradation phenomenon appears to be negligible on the time scale of the experiment described in Fig. 3. Indeed a two-fold increase of nitrogenase concentration occurred within 2 h after a shift-up of light intensity (Table II). This effect must be attributed to an increase of the rate of nitrogenase synthesis.

From the theoretical laws of continuous culture [20], the rate of synthesis k and the concentration E of nitrogenase vary according to the equation

$$dE/dt = k - DE,$$

where D is the dilution rate. At steady state ($dE/dt = 0$), the rate of nitrogenase synthesis is proportional to the enzyme concentration ($k = DE$). The experiments of Fig. 1 and Fig. 2 show that the intracellular nitrogenase concentration is a function of the light intensity ($E = f(I)$). Hence, at each steady state the nitrogenase concentration reflects the rate of enzyme synthesis, which is therefore light dependent. This suggests that the control of nitrogenase synthesis by light might be energetic, the extent of nitrogenase derepression being a function of the available energy to the bacteria. Reidl et al. [21] observed that the capacity of photophosphorylation of *Rps. capsulata* cells depends on the light intensity to which cells were exposed during growth. This capacity, measured as the maximal rate of phosphorylation per membrane protein, was found to be 1.5-fold higher in cells grown under high light than in cells grown under low light intensity. Although comparison of these results with ours may not be valid due to differences in experimental conditons, the question arises whether the rate of nitrogenase synthesis is dependent on the rate of photophosphorylation.

The preferential synthesis of nitrogenase over other cellular constituents indicates that, under *N*-deficient conditions and high illumination, the transcription of *nif* genes is favoured. The ratio Rc1/Rc2 remained unchanged and close to one, suggesting that the nitrogenase components are cosynthesized. In agreement with this observation, the structural genes coding for the nitrogenase subunits have been found to be organized in a single operon in *Rps. capsulata* [22].

The intermediary metabolite guanosine tetraphosphate (ppGpp) which would enhance the transcription of *nif* genes has been postulated to be involved in the regulation of *nif* expression in *Klebsiella pneumoniae* [23] and in *Rps. palustris* [24]. In *Rps. palustris* a correlation was indeed observed between derepression of nitrogenase and a rise of the ppGpp pool size and conversely. However, other authors have shown that the ppGpp level increased after a down shift of light

intensity in *Rhodopseudomonas sphaeroides* [25], a treatment which was found to repress nitrogenase synthesis in *Rps. capsulata* [12]. On the other hand, in *K. pneumoniae*, Nair and Eady [26] measured enhanced levels of *nif* expression without accumulation of ppGpp. These results are clearly contradictory and further work is required to determine whether ppGpp or another intermediary compound is implicated in the activation of *nif* genes transcription.

Acknowledgements

The authors thank Dr J.C. Willison for critical reading of the manuscript. This work was supported by research grants from the Centre National de la Recherche Scientifique (CNRS, APP PIRSEM) and the Commission of the European Communities (EEG ESD-F-018).

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