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Changes in the NAD(P)H concentration caused by addition of nitrogenase 'switch-off' effectors in *Rhodospirillum rubrum* G-9, as measured by fluorescence

Agneta Norén, Stefan Nordlund*

Department of Biochemistry, Arrhenius Laboratories for Natural Science, Stockholm University, S-106 91 Stockholm, Sweden

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Abstract The effect of nitrogenase 'switch-off' effectors on the concentration of NAD(P)H in *Rhodospirillum rubrum* G-9 was investigated by fluorescence. A rapid decrease in fluorescence was observed when cells, either N_2-grown or nitrogen-starved, were subjected to the effectors, but not when sodium chloride or Tris buffer was added. No effects on the fluorescence were observed in non-nitrogen fixing cultures except when NAD^+ was added. The results strongly indicate that the redox state of the pyridine nucleotide pool affects the control of the regulation of nitrogenase activity in *R. rubrum*.

Key words: Nitrogen fixation; Switch-off effect; *Rhodospirillum rubrum*; $NAD(P)^+$ concentration

1. Introduction

Nitrogen fixation is carried out by a number of bacteria, in a reaction catalyzed by nitrogenase, which consists of two proteins, dinitrogenase and dinitrogenase reductase. Electrons are transferred from dinitrogenase reductase to dinitrogenase in a reaction requiring hydrolysis of MgATP [1]. In a number of phototrophs and some species of *Azospirillum*, nitrogen fixation is regulated not only genetically but also metabolically [2,3]. In the photosynthetic bacterium *R. rubrum*, nitrogenase activity is regulated by reversible inhibition, a phenomenon referred to as the 'switch-off' effect [4]. At the molecular level this effect is due to reversible modification of dinitrogenase reductase by ADP-ribosylation of one of its two identical subunits on an arginine residue, Arg-101, when the cells are subjected to darkness, ammonium ions, glutamine, asparagine or oxygen [2,3]. Other switch-off effectors are carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and phenazine methosulphate (PMS) [5]. The modification of dinitrogenase reductase is catalyzed by dinitrogenase reductase ADP-ribosyl transferase (DRAT) with NAD^+ as the donor of ADP-ribose [2]. The reverse reaction is catalyzed by dinitrogenase reductase activating glycohydrolase (DRAG) [2], which requires ATP and a divalent cation such as manganese or ferrous iron [6].

The internal signal between the switch-off effector and DRAG/DRAT has not yet been identified, but the nitrogen status and the $NAD(P)^+$/NAD(P)H ratio have been suggested to be involved in the regulation of these enzymes. We have previously shown that adding NAD^+ to a nitrogen-fixing culture of *R. rubrum* results in a reversible decrease in activity, an effect dependent on light intensity; at lower light intensities the effect is more pronounced [7]. The effect of NAD^+ can also be seen in nitrogen-starved cells which cannot be 'switched off' by any of the other effectors tested. We have previously suggested that an increase in the NAD^+ concentration could be involved in the control of the activities of DRAG and especially DRAT, and that the nitrogen status of the cell is also of possible importance [7]. An increase in the NAD^+ concentration could also act as a direct signal for DRAT activity since the enzyme is NAD^+ dependent, having a high K_m for NAD^+ with dinitrogenase reductase from *R. rubrum* [2,7].

In this investigation we have studied the influence of switch-off effectors on the NAD(P) pool, by measuring NAD(P)H fluorescence when cultures were subjected to ammonia, darkness, oxygen, NAD^+ or glutamine.

2. Materials and methods

R. rubrum, strain G-9, was grown photoheterotrophically in the medium of Ormerod et al. [8] with the omission of glutamate under an atmosphere of N_2/CO_2 (95:5). Nitrogen starvation was obtained by flushing the culture with argon for 12–16 h before assay.

Fluorescence was monitored continuously, with the excitation at 350 nm and the emission recorded at 457 nm. The actinic light was filtered with a 530-nm filter. 3 ml cell suspension in 5 ml stoppered anaerobic cuvettes with an atmosphere of nitrogen were used, with constant stirring and illumination, except when otherwise indicated.

Additions of anaerobic solutions of switch-off effectors were made directly into the anaerobic cuvette during fluorescence monitoring, using microliter syringes.

Nitrogenase activity was measured as the reduction of acetylene using 2 ml cell suspensions [9]. The reaction was run for 15 min.

3. Results and discussion

In these investigations, *R. rubrum* G-9, a blue-green mutant devoid of carotenoids with absorbance maxima in the region of NAD(P)H fluorescence, was used. In the wild-type *R. rubrum*, nitrogenase 'switch-off' is a well-established phenomenon, but strain G-9 has not been studied in this respect. However, as shown in Table 1, addition of switch-off effectors to cultures of G-9 resulted in inhibition to a degree essentially the same as in the wild-type. Sodium chloride had no effect.

To establish conditions for fluorescence studies, emission spectra for NADH in Tris buffer and in a cell suspension of *R. rubrum* G-9 were recorded. As shown in Fig. 1 the spectra are essentially the same. Also shown is the effect of an addition of NADH.

To study the effect of light on the NAD(P)H concentration,

*Corresponding author. Fax: (46) 8 15 77 94.
E-mail: stefan@biokemi.su.se

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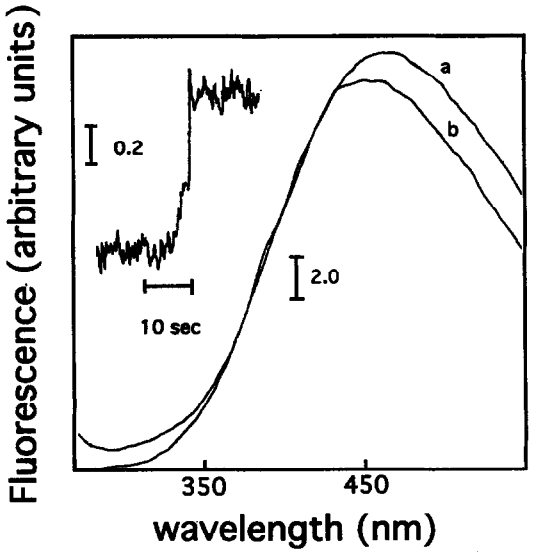

Fig. 1. Fluorescence spectra of NADH. Addition of 100 μM (final concentration) NADH to (a) 100 mM Tris buffer, pH 7.8, and (b) a cell suspension of *R. rubrum* G-9. The insert shows the change in fluorescence caused by the addition of 100 μM (final concentration) NADH to a suspension of *R. rubrum* G-9.

cells were incubated in the dark for 15 min and then subjected to light. This resulted in an increase in fluorescence (data not shown). Addition of oxygen caused a decrease in fluorescence, whereas addition of nitrogen had no effect (data not shown). These effects are in good agreement with previous studies reported by Jackson and Crofts [10], and clearly demonstrate that the redox state of the pyridine nucleotide pool is dependent on the photosynthetic system as well as the respiratory activity.

The best established switch-off effector in *R. rubrum* is ammonium ions, which cause a rapid decrease in nitrogenase activity when added to N_2-grown cultures of *R. rubrum* [2,3], but not in nitrogen-starved cultures unless glutamate is added prior to ammonium ions. However, both in N_2-grown and nitrogen-starved cultures addition of ammonium ions caused a decrease in fluorescence (Fig. 2a,b), indicating that the concentration of reduced pyridine nucleotides is changed in both types of cultures. Addition of the same volume and concentration of sodium chloride or Tris buffer did not lead to a significant change in fluorescence (data not shown). Addition of ammonium ions to an NH_4^+-grown, nitrogen non-fixing culture did not affect the fluorescence (data not shown). Earlier studies in another photosynthetic bacterium, *Rhodopseudomonas sphearoides*, have established that the addition of ammonium ions does not affect the membrane potential to any significant extent [11], suggesting that the observed change in NAD(P)H concentration is probably due to some other mechanism. In diazotrophically grown *R. rubrum*, ammonium ions are assimilated through the glutamine synthetase/glutamate synthase pathway, in which NADPH is required [3,12]. The addition of NH_4^+ is postulated to momentarily increase the flow through these reactions, thus leading to a decrease in the concentration of reduced pyridine nucleotides, possibly mainly NADH, through the action of transhydrogenase.

The fact that addition of NH_4^+ to nitrogen-starved cells also produced a decrease in fluorescence, although no 'switch-off' of nitrogenase is obtained under these conditions, indicates that a change in the $NAD(P)^+$ concentration alone is not sufficient to affect the control of DRAG/DRAT. It is also possible that under these limiting growth conditions the total concentration of pyridine nucleotides is too low to give concentrations of

Table 1
Nitrogenase 'switch-off' in *Rhodospirillum rubrum*, strain G-9

Addition	Concentration (mM)	Nitrogenase activity (%)[a]
None	–	100
NH4Cl	2	11
Glutamine	3	47
NAD^+	5	13
NaCl	5	103

[a] 100% = 198 nmol ethylene/h · ml.

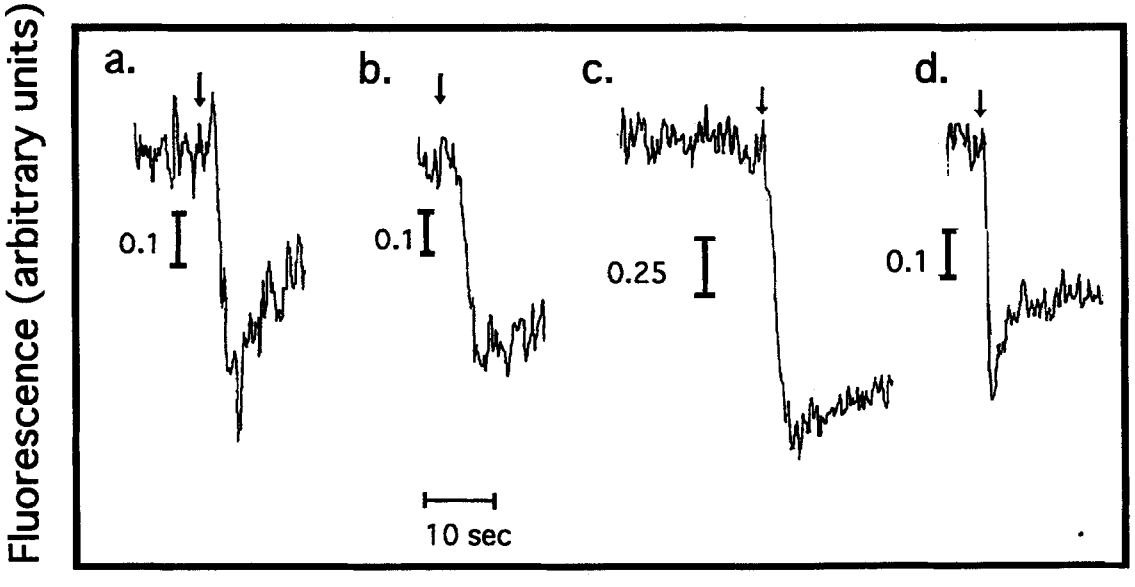

Fig. 2. Changes in fluorescence caused by addition of switch-off effectors to *R. rubrum* G-9. Additions: a,b, ammonium chloride, final concentration 7.5 mM; c, glutamine, final concentration 5 mM; d, NAD^+, final concentration 2.5 mM. Growth conditions: a,c,d, N_2-grown; b, nitrogen-starved.

$NAD(P)^+$ high enough to affect the control. The effect of glutamine was also investigated in N_2-grown (Fig. 2c) and nitrogen-starved cells (data not shown): the changes in fluorescence were essentially as those obtained with ammonium ions. The decrease in fluorescence can also, in this case, be explained by an increased flux through the reaction catalyzed by glutamate synthase, leading to an increase in the concentration of $NADP^+$, and via the action of transhydrogenase to a corresponding increase in $[NAD^+]$.

We have previously shown that when adding NAD^+ to a nitrogen-fixing culture of *R. rubrum* there is an instant decrease in activity, an effect also obtained in nitrogen-starved cells [7]. The results depicted in Fig. 2d show that addition of NAD^+ causes a decrease of fluorescence in N_2-grown cells and the same effect was observed in NH_4^+-grown as well as in nitrogen-starved cells (data not shown). The mechanism(s) behind these effects is not clear since oxidation of the internal NAD(P)H pool by the NAD^+ added should lead to no, or very little change in fluorescence. One possible explanation for the observed decrease in fluorescence is that added NAD^+ is reduced in the periplasm, thereby oxidizing the internal pool of NAD(P)H, and then reoxidized by as yet unidentified reactions in this cell compartment.

In conclusion, this investigation clearly provides evidence for an oxidation of the intracellular pool of reduced pyridine nucleotides upon addition of switch-off effectors to diazotrophically grown *R. rubrum*. The results thus support the hypothesis that an increase in the concentration of NAD^+ is one of the factors controlling the activities of DRAG and DRAT. However, the fact that addition of ammonium ions or a shift to darkness does not lead to switch-off in nitrogen-starved cells, although there is a decrease in the NAD(P)H concentration, necessitates postulation that yet other factors are required in this control system. Most likely, the nitrogen status of the cell is one of them.

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