The role of NAD^+ as a signal during nitrogenase switch-off in *Rhodospirillum rubrum*

Agneta NORÉN, Abdelhamid SOLIMAN and Stefan NORDLUND*

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

The role of NAD^+ in the metabolic regulation of nitrogenase, the 'switch-off' effect, in *Rhodospirillum rubrum* has been studied. We now show that the decrease in nitrogenase activity upon addition of NAD^+ to *R. rubrum* is due to modification of dinitrogenase reductase. There was no effect when NAD^+ was added to a mutant of *R. rubrum* devoid of dinitrogenase reductase ADP-ribosyltransferase, indicating that NAD^+ 'switch-off' is an effect of the same regulatory system as ammonium 'switch-off'. We also show that oxaloacetate and α-ketoglutarate function as 'switch-off' effectors. On the other hand β-hydroxybutyrate has the opposite effect by shortening the 'switch-off' period. Furthermore, by using an inhibitor of glutamate synthase the role of this enzyme in 'switch-off' was investigated. The results are discussed in relation to our proposal that changes in the concentration of NAD^+ are involved in initiating 'switch-off'.

INTRODUCTION

Nitrogen fixation in a number of photosynthetic bacteria and in some species of *Azospirillum* is regulated on the metabolic level [1], in addition to the transcriptional control operating in all diazotrophs studied. In photosynthetic bacteria, the metabolic regulation is manifested as a decrease in nitrogenase activity when certain compounds are added or when cells are shifted to darkness. This effect has been termed 'switch-off' [2] and a number of 'switch-off' effectors have been reported, although ammonium ions, glutamine, asparagine, oxygen and shift to darkness are physiologically the most relevant ones in phototrophs [1,3].

'Switch-off' is the effect of the reversible inactivation of dinitrogenase reductase, one of the two proteins constituting the nitrogenase complex, due to covalent modification by an ADP-ribose moiety, attached to a specific arginine residue (Arg-101) in one of the subunits of dinitrogenase reductase [4]. This reaction is catalysed by dinitrogenase reductase ADP-ribosyltransferase (DRAT), whereas the reverse reaction, i.e. reactivation of dinitrogenase reductase, is due to hydrolytic removal of the ADP-ribose moiety in a reaction catalysed by dinitrogenase reductase activating glycohydrolase (DRAG) [1]. The modification of dinitrogenase reductase can be conveniently demonstrated, as the ADP-ribosylated subunit has a slower migration on SDS/PAGE than the unmodified form [1].

One of the major questions concerning the 'switch-off' phenomenon has been the identity of the metabolic signal between the 'switch-off' effector and the enzymes of the regulatory cycle, i.e. DRAT and DRAG. Studies of changes in amino acid and nucleotide pools have been reported but have not given a conclusive answer [5–8]. A role of the ammonium assimilatory pathway, i.e. glutamine synthetase and glutamate synthase, has also been proposed, and most recently it was shown that a mutant of *Rhodospirillum rubrum* lacking NtrC did not respond either as fast or to the same extent as the wild-type upon addition of ammonium ions [9].

We have previously provided evidence for a role of changes in the concentration of NAD^+ in the 'switch-off' effect, by showing that addition of NAD^+ to diazotrophically grown *R. rubrum* leads to a reversible decrease in nitrogenase activity [10]. We have also demonstrated that addition of 'switch-off' effectors to a culture of *R. rubrum* will indeed cause a decrease in the NADH concentration, supporting a role for NAD^+ in the molecular signalling leading to 'switch-off' [11].

In this paper we demonstrate that addition of NAD^+ leads to modification of dinitrogenase reductase and that NAD^+ does not have any effect when added to a *R. rubrum* mutant lacking DRAT. We also report on further studies of compounds that cause 'switch-off' or influence this effect and that can be related to changes in the oxidation state of NAD in *R. rubrum*.

MATERIALS AND METHODS

R. rubrum, Strain S1, was grown photoheterotrophically, to A_{600} = 1.5–2, under an atmosphere of N_2/CO_2 (95:5) in the medium of Ormerod et al. [12], with the omission of ammonium sulphate. *R. rubrum* UR 212, a mutant lacking DRAT [13], was grown in the same medium but with the addition of kanamycin (10 μg/ml) and streptomycin (100 μg/ml).

Nitrogenase activity was determined as acetylene reduction as described previously [5]. Cells were incubated in anaerobic vials with 10% acetylene and effector(s) added as indicated in each experiment. Gas samples were removed at the times shown and the ethylene produced was assayed by gas chromatography.

Modification of dinitrogenase reductase was assayed by a combination of SDS/PAGE [14] and immunoblotting [15]. Degassed SDS sample buffer [2 ml; 130 mM Tris, pH 6.8/4.2% (w/v) SDS, 20% (v/v) glycerol, 0.003% (w/v) Bromophenol Blue and 10% (v/v) 2-mercaptoethanol] was added to 2 ml whole cell samples. Mixtures were incubated at 30 °C for 60 s, boiled for 30 s, and centrifuged at 16000 ***g*** for 5 min [16]. Supernatants were removed and stored at −80 °C until use. This extraction procedure minimizes the change in modification of dinitrogenase reductase due to long incubation in darkness, which would increase modification, or longer periods of heating, which would lead to release of the ADP-ribose moiety. Thawed samples were loaded on to 12% (w/v) acrylamide gels with low cross-linker concentration, acrylamide/bisacrylamide 171:1 [17]. Transfer of

Abbreviations used: DRAT, dinitrogenase reductase ADP-ribosyltransferase; DRAG, dinitrogenase reductase activating glycohydrolase; DON, 6-diazo-5-oxo-L-norleucine.

* To whom correspondence should be addressed.

proteins to a PVDF membrane was performed in 192 mM glycine/25 mM Tris, pH 8.3/20% methanol for 2 h at 250 mA. Polyclonal antibodies against *R. rubrum* dinitrogenase reductase, diluted 1:3000, and horseradish peroxidase-coupled secondary antibody (1:5000) were used for enhanced chemiluminescence detection of proteins according to manufacturer's instructions.

RESULTS AND DISCUSSION

In our previous reports we have shown that NAD^+, when added *in vivo*, does lead to a decrease in nitrogenase activity, although the molecular mechanism has not been established. In order to demonstrate that this inhibition is due to a modification of dinitrogenase reductase, NAD^+ was added to N_2-grown *R. rubrum* and samples were removed for SDS/PAGE. The results shown in Figure 1(A) clearly demonstrate that dinitrogenase reductase is in fact modified, most likely by ADP-ribosylation, as the intensity of the upper band, a modified subunit of dinitrogenase reductase, increases (lane 2) in a way similar to that after addition of ammonium ions (lane 3). Figure 1(B) shows that the inhibition by NAD^+ is reversible and that the change in activity parallels the change in the amount of the upper, modified band. To provide further evidence that 'switch-off' caused by addition of NAD^+ is due to the same molecular events as with ammonium ions as 'switch-off' effector, NAD^+ was added to a N_2-fixing culture of a *R. rubrum* mutant lacking DRAT [13]. In this case no decrease in activity and no modification of dinitrogenase reductase could be demonstrated (Figure 2).

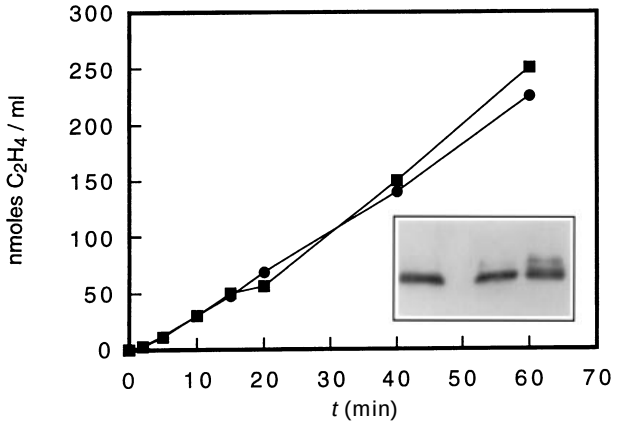

Figure 2 Effect of NAD^+ on modification and activity in the mutant *R. rubrum* UR212

The experiment was run as described in the Materials and methods section. Samples for activity measurements were taken at the times shown in the Figure: (●) no addition; (■) 5 mM NAD^+, added to UR212. The inset shows for comparison SDS/PAGE of: lane a, 5 mM NAD^+ added to UR212; lane b, no addition to *R. rubrum* wild-type; lane c, 5 mM NAD^+ added to wild-type. Samples were taken 10 min after the addition.

These results, together with our previous demonstration that addition of ammonium ions or shift to darkness lead to a decrease in the NAD(P)H concentration in *R. rubrum* [11], clearly support the hypothesis that changes in the NAD^+ concentration play a central role in the signalling to the DRAT/DRAG regulatory cycle. The fact that DRAT has a high K_m value, 2 mM, for NAD^+ with dinitrogenase reductase from *R. rubrum in vitro* [1], lends further support to this proposal.

Under diazotrophic conditions ammonium ions are assimilated via the reactions catalysed by glutamine synthetase and glutamate synthase. The latter reaction leads to the production of glutamate from α-ketoglutarate and glutamine with NADPH being oxidized to $NADP^+$. When ammonium ions are added as 'switch-off' effector glutamine is formed, in the reaction catalysed by glutamine synthetase, increasing the flux through the glutamate synthase reaction, which is postulated to lead to a transient increase in the $NADP^+$ concentration. The reaction catalysed by transhydrogenase leads to the reduction of $NADP^+$ by NADH. The reaction is dependent on a proton gradient across the chromatophore membrane [18]. In summary, addition of ammonium ions would lead to an increase in the NAD^+ concentration. The involvement of glutamine synthetase has been demonstrated previously in studies using methionine sulphoximine, an inhibitor of this enzyme [19].

The reaction catalysed by glutamate synthase is thus of central importance. Inhibitors of this enzyme have been used in studies of the 'switch-off' effect, with somewhat contradictory results. Li et al. [8] reported 'switch-off' by addition of 200 μM azaserine to an ammonia-limited culture of *R. rubrum*, whereas Kanemoto and Ludden [6], using a 1 mM concentration of this inhibitor, could not correlate the decrease in nitrogenase activity with the ADP-ribosylation of dinitrogenase reductase. It was suggested that the decrease in nitrogenase activity was due to a decrease in total dinitrogenase reductase present [1,6]. Another inhibitor of

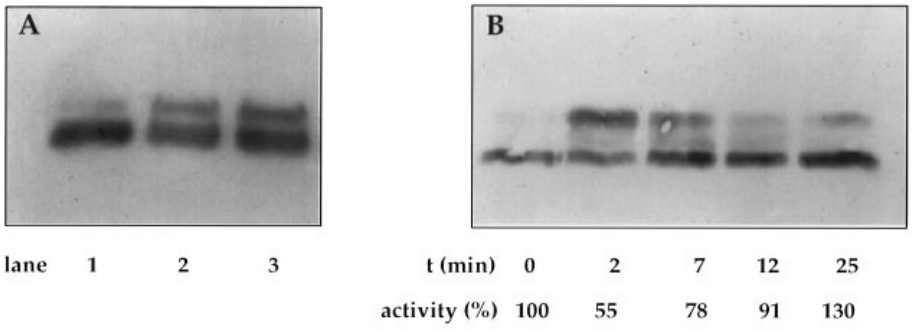

Figure 1 Modification of dinitrogenase reductase during 'switch-off'

(**A**) The experiment was run as described in the Materials and methods section and samples for SDS/PAGE were taken after 5 min. The additions were: lane 1, none; lane 2, 5 mM NAD^+; lane 3, 1 mM NH_4^+. (**B**) The experiment was run as described in the Materials and methods section: 5 mM NAD^+ was added at time zero. 100% activity = 300 nmol of $C_2H_4 \cdot ml^{-1} \cdot h^{-1}$. Samples for SDS/PAGE and activity measurements were taken at the times shown in the Figure.

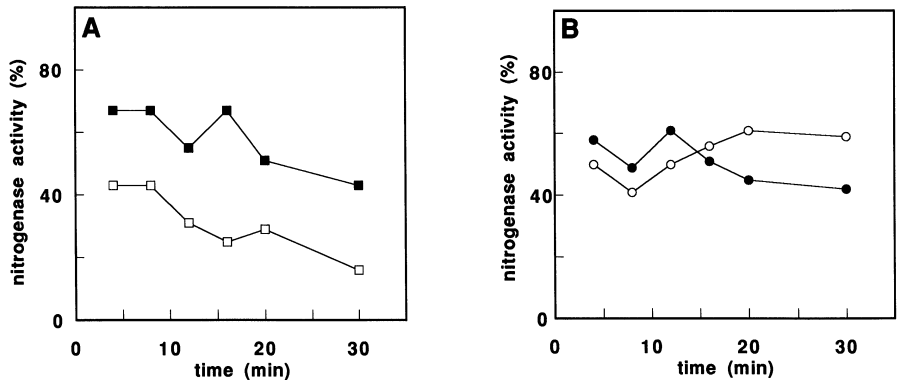

Figure 3 Effect of DON on 'switch-off' by NAD^+ and glutamine in N_2-grown *R. rubrum*

Cells were grown in 1 litre cultures and all data shown in the Figure are from the same culture. Samples (2 ml), one for each time point, were transferred to a 25 ml injection vial containing NAD^+, glutamine or, for the controls, no addition and acetylene. At each time point the reaction was stopped by addition of 1 ml of perchloric acid (20%, v/v) and the samples were assayed for ethylene produced. After the first series, DON was added to the 1 litre culture. After 10 min incubation a new series of assays was started and run as the first one. Activities are given as the percentage of the vials run without effector added. Open symbols: DON not added. Solid symbols: 5 μM DON added: (**A**) 0.5 mM glutamine; (**B**) 2 mM NAD^+.

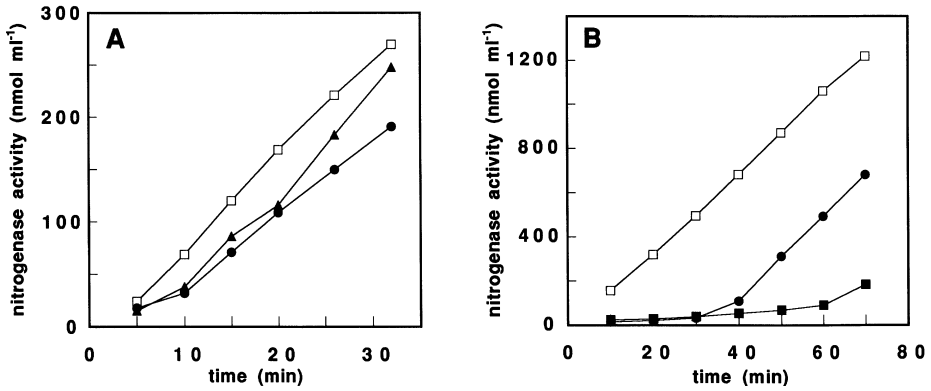

Figure 4 'Switch-off' by oxaloacetate and α-ketoglutarate, and the effect of β-hydroxybutyrate on 'switch-off' in N_2-grown *R. rubrum*

Cells were grown and nitrogenase activity was assayed as described in the Materials and methods section: (**A**) □, control; ▲, 4 mM oxaloacetate; ●, 4 mM α-ketoglutarate; (**B**) □, control; ■, +0.25 mM NH_4^+; ●, +0.25 mM NH_4^+/20 mM β-hydroxybutyrate.

glutamate synthase, 6-diazo-5-oxo-L-norleucine (DON), has also been demonstrated to cause inhibition of nitrogenase activity in glutamate-grown *R. rubrum* [20] at low light intensities.

We have investigated the effect of DON on 'switch-off' by glutamine and NAD^+ respectively. The results depicted in Figure 3 show that DON (5 μM) did not affect 'switch-off' by NAD^+, but markedly slowed down the effect of glutamine. Since the concentration of DON used in this experiment inhibits purified glutamate synthase from *R. rubrum* [21], we interpret these results as evidence for a role of glutamate synthase in 'switch-off' by producing oxidized NADP. However, we did also, as the previous investigators, observe some inhibition (35%) of nitrogenase activity by DON alone, which may be caused by pleiotropic effects in the cell, since DON and azaserine are general amidotransferase inhibitors.

In order to induce changes in the NAD^+/NADH ratio without using effectors containing nitrogen, oxaloacetate or α-ketoglutarate was added to N_2-grown cultures of *R. rubrum*. The rationale was that addition of oxaloacetate would lead to oxidation of NADH in the reaction catalysed by malate dehydrogenase, whereas α-ketoglutarate would increase the rate of the glutamate synthase reaction. As shown in Figure 4(A), both of these additions did lead to 'switch-off'. Similar results have been reported from studies on *Rhodobacter capsulatus* [22].

If an increase in the concentration of NAD^+ is involved in the 'switch-off' effect, increasing the metabolic capacity in the cell to reduce NAD^+ should have the opposite effect, i.e. decrease 'switch-off' and/or increase the rate of 'switch-on'. To investigate this hypothesis we studied the effect of β-hydroxybutyrate, which will reduce NAD^+ in the reaction catalysed by β-hydroxybutyrate dehydrogenase, which has been demonstrated in ammonia-grown *R. rubrum* [23] and also in N_2-grown cells (A. Soliman, unpublished work). As shown in Figure 4(B), addition of β-hydroxybutyrate clearly shortens the 'switch-off' period, indicating that indeed reduction of NAD^+ is required for 'switch-on' to occur.

The effects reported in this paper can all be explained by the involvement of changes in the cellular NAD^+ concentration in the metabolic regulation of nitrogenase activity. Appointing a central role to changes in the redox state of NAD does also offer an explanation of the action of other 'switch-off' effectors, e.g. darkness and oxygen [17]. With respect to these two effectors, it has been shown that in *R. rubrum*, under anaerobic conditions in the light, NAD and NADP are present in the reduced form at 70

and 100% respectively [24]. These authors also showed that upon shifting cells to darkness these nucleotides were oxidized within 1 min and that the same effect was obtained when the cells were treated with oxygen. Furthermore, we have reported that darkness does have the same effect on the NAD(P)H concentration in N_2-grown cells [11].

However, although assigning a key role to changes in the redox state of NAD as a signal between 'switch-off' effectors and the DRAT/DRAG regulatory cycle could account for most of the effects reported here and by others, one major issue remains unexplained, the regulation of DRAG. The effect of NAD^+ on DRAT can be clearly understood, but so far no major effect of either oxidized or reduced NAD(P) on DRAG has been demonstrated [11]. A more complete understanding of the metabolic regulation of nitrogenase requires that effectors of both DRAT and DRAG are identified.

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