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Electron transport to nitrogenase in *Rhodospirillum rubrum*: Role of energization of the chromatophore membrane *

Anders Lindblad & Stefan Nordlund 1
Department of Biochemistry, Arrhenius Laboratories for Natural Sciences, Stockholm University, S-106 91
Stockholm, Sweden; 1Author for correspondence

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Abstract

Nitrogen fixation is dependent on a source of ATP and the generation of a reductant at low enough red-ox potential to transfer electrons to nitrogenase. In *Rhodospirillum rubrum*, grown photoheterotrophically, ATP is produced by photophosphorylation, a process studied in great detail, but the source of reductant for nitrogenase is as yet unidentified. In this report we have studied the effect on nitrogen fixation when the energization of the chromatophore membranes was changed, by decreasing the light intensity or by addition of uncouplers. When the light intensity was lowered a pronounced decrease in nitrogenase activity was observed although there was no decrease in the ATP/ADP ratio. The inhibition observed was not due to ADP-ribosylation, as the same effect was observed in a mutant devoid of the enzymes in the metabolic regulatory cascade operating in *R. rubrum* and some other diazotrophs. Even at low concentrations of the uncouplers used, a drastic decrease in the ATP/ADP ratio was observed. However, this decrease in the ATP/ADP ratio did not cause a decrease in nitrogenase activity. At higher concentrations of uncouplers, nitrogenase activity decreased but the ATP/ADP ratio remained essentially at a constant low level. These results support a model in which reduction of the electron donor(s) to nitrogenase in *R. rubrum* is coupled to the energization of the chromatophore membranes.

Abbreviations: CCCP – carbonyl cyanide *m*-chlorophenyl hydrazone; FCCP – carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone; TTFB – 4,5,6,7-tetrachloro-2-trifluoromethyl benzimidazol; DRAT – dinitrogenase reductase ADP-ribosyl tranferase; DRAG – dinitrogenase reductase activating glycohydrolase

Introduction

Biological nitrogen fixation is a strictly prokaryotic phenomenon which, in all systems hitherto examined, is catalysed by the nitrogenase protein complex. Nitrogenase consists of two proteins, dinitrogenase reductase (*Fe*-protein) and dinitrogenase (*MoFe*-protein) (Burris 1991). Dinitrogenase reductase is constituted of two identical subunits, encoded by *nifH*, bridged by one [4Fe–4S] cluster. Dinitrogenase is an \( \alpha_2\beta_2 \) tetramer, encoded by *nifD* and *nifK* respectively, which contains two Fe-Mo cofactors (FeMoco) and two [4Fe–4S] clusters (P-clusters) (Kim et al. 1994). Nitrogen fixation is an energetically costly process, at least 16 ATP and 8 electrons are required for each dinitrogen molecule reduced (Burris 1991). The expression of genes, encoding polypeptides involved in nitrogen fixation, *nif* genes, are transcriptionally regulated by the nitrogen status in the cell (Merrick 1992). In addition, the activity of nitrogenase is metabolically regulated in some diazotrophs, e.g. *Rhodospirillum rubrum*, by reversible ADP-ribosylation of one of the subunits of dinitrogenase reductase, referred to as the ‘switch off’ effect (Ludden et al. 1989). Addition of ‘switch-off’ effectors, such as ammonium ions, glutamine or subjecting cells darkness, leads to ADP-
nitrogenase activity requires a supply of both ATP and a strong reductant. It has, however, been established that the energy charge in diazotrophs fixing dinitrogen is low, compared to growth under N-rich conditions (Ludden 1991). Furthermore, it has been shown that R. rubrum can grow under dark fermentative conditions indicating that photosynthesis as such is not required for nitrogen fixation (Schultz et al. 1985).

The pathway generating reductant to nitrogenase has been well characterised only in the enterobacterium Klebsiella pneumoniae. In this organism a pyruvate: flavodoxin oxidoreductase, encoded by nifJ, has been shown to transfer electrons from pyruvate to nitrogenase via a nitrogen fixation specific flavodoxin, encoded by nifF (Ludden 1991).

In contrast, very little is known about the electron transport pathway to nitrogenase in photosynthetic diazotrophs. These bacteria represent a different metabolic strategy with respect to energy and reductant metabolism. A direct involvement of the photosynthetic machinery in production of reducing equivalents to nitrogenase is not plausible as the primary photosynthetic acceptor has a midpoint potential too positive to function as a reductant to nitrogenase.

In the photosynthetic bacterium R. rubrum, pyruvate dependent nitrogenase activity has been demonstrated in vivo (Ludden et al. 1981) and a pyruvate oxidoreductase from R. rubrum was purified and partially characterized (Brostedt et al. 1991). This enzyme shows high degree of similarity to the enzyme from K. pneumoniae with respect to molecular and substrate properties. The gene encoding the pyruvate oxidoreductase from R. rubrum, including an upstream region, was cloned and sequenced (Lindblad et al. 1996). The deduced amino acid sequence of the nifJ-like gene in R. rubrum was compared to published sequences from K. pneumoniae and Anabaena 7120. The total percentage of identity of the R. rubrum sequence was 47% to K. pneumoniae and 56% to Anabaena 7120. A probable σ54 promoter was identified in the upstream region of the nifJ-like gene from R. rubrum (Lindblad et al. 1996).

A nifJ mutant was constructed to investigate the role of NIFJ in the electron transport pathway in R. rubrum. This mutant (SNJ-1) showed the same growth rates under all conditions studied, including diazotrophy, as the wild-type. The ability of the mutant to grow under nitrogen fixing conditions indicates that an alternative electron transport pathway is present in R. rubrum (Lindblad et al. 1996).

It has previously been suggested that the membrane potential is involved in reducing electron carriers to nitrogenase in different diazotrophs. Whole cell nitrogenase activity of Rhodobacter sphaeroides could be inhibited by lowering the potential across the cytoplasmic membrane without changing the ATP/ADP ratio (Haaker et al. 1982). The effect of ionophores was also investigated in bacteroids of Rhizobium leguminosarum. (Laane et al. 1979). In this study, treatment of bacteroids with low concentrations of valinomycin and nigericin led to inhibition of nitrogenase activity although the ATP/ADP ratio remained high. Similar results have been obtained with Azotobacter vinelandii, using TTFB as uncoupler (Haaker et al. 1974).

A new class of nitrogen fixation genes in Rhodobacter capsulatus has been identified by Klipp and coworkers (Schmehl et al. 1993). Some of these rnf genes have been suggested to encode membrane spanning proteins involved in the electron transport to nitrogenase. Mutant studies of the rnf genes have further supported this hypothesis.

In this study we report on the effects of light intensity and uncouplers on the ATP/ADP ratio, nitrogenase activity and modification of the dinitrogenase reductase in R. rubrum. Two different strains of R. rubrum were used, wild-type and a mutant lacking the ability to ADP-ribosylate dinitrogenase reductase. These studies indicate a relationship between membrane energization and nitrogenase activity. A model for the electron transport to nitrogenase in R. rubrum is proposed.

Materials and methods

Growth of cells

Two different strains of R. rubrum were used: wild-type, S1, and UR212 a draT mutant lacking both DRAT and DRAG activity (Zhang et al. 1995). R. rubrum, strains S-1 and UR212, were grown photo-heterotrophically in the medium of Ormerod and coworkers (Ormerod et al. 1961), with the omission of fixed nitrogen, under an atmosphere of N2/CO2(95:5%). Streptomycin, 100 μg/ml, and kanamycin, 20 μg/ml, were added to UR212 cultures.

Sample preparation and nitrogenase activity assay

Two ml of cells were incubated at 30 °C for 10 min, in 25 ml evacuated vials flushed with argon. Light intensity was varied with a potentiometer coupled to the