Nitrogen Fixation and Nitrate Respiration by *Azospirillum brasilense*

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The O_2- sensitivitiy of N_2-fixation by the carotenoid forming strain *Azospirillum brasilense* Cd and the colourless strain Sp 7 is compared in the present communication. As no difference in the reaction is observed with both strains, it is concluded that carotenoids do not protect nitrogenase from damage by O_2. *Azospirillum* spp. have also been shown to perform NO_3^-- dependent N_2-fixation. The physiological properties of this reaction are described in more detail in the present communication. Evidence is presented that NO_3^-- dependent N_2-fixation is a transitory reaction, proceeding only as long as the enzymes of assimilatory nitrate reduction are synthesized by the cells.

Introduction

The genus *Azospirillum* is remarkable with respect to its metabolism of inorganic nitrogen. These bacteria bring about N_2-fixation, assimilatory NO_3^--reduction, respiratory reduction of NO_3^- to NO_2^-, N_2O, N_2 or NH_4^+, and NO_3^--dependent N_2-fixation [1]. They deserve special attention also for practical reasons because they live in association with roots of grain crops and forage grasses. Depending in particular on the O_2 and NO_3^- concentrations and on the availability of organic carbon in soils, *Azospirillum* spp. may either enrich the nitrogen content by N_2-fixation or liberate nitrogen gas from the soil or roots by denitrification. The physiological transformations of inorganic nitrogen by *Azospirillum* spp. and the interference of the pO_2 in these reactions have not been fully elucidated. A better understanding of the physiological processes is, however, prerequisital to assess the ecological role of the plant-*Azospirillum* association. Two sets of physiological experiments will be reported in the present communication to get more informations about the metabolism of inorganic nitrogen by *Azospirillum* spp.

First, Nur *et al.* [2] have recently reported that a red pigmented strain of *A. brasilense*(strain Cd) produces carotenoids specifically under aerobic conditions. They suggested from indirect experimental evidence that the carotenoids play a role in the protection of nitrogenase from damage by O_2. The ability to synthesize carotenoids may, therefore, enable such strains to perform N_2-fixation under all O_2-tensions in the soil in contrast to the colourless strains which have developed poor mechanisms to protect their nitrogenase. If so, carotenoid-forming strains of *Azospirillum* would be of great interest for practical applications. Nur *et al.* [2], however, did not directly determine the O_2-sensitivity of N_2-fixation. The effect of O_2 on C_2H_2-reduction is tested side by side in *A. brasilense* Cd and the nonpigmented strain Sp 7 in the present communication.

Second, NO_3^- has been shown to support nitrogenase activity of *Azospirillum* spp. under strictly anaerobic conditions [3, 4]. These findings suggested that NO_3^- serves as the terminal respiratory electron acceptor, and that NO_3^--respiration provides enough energy to support N_2-fixation as it had also been described for *Rhizobium* bacteroids [5]. In contrast, Nelson and Knowles [6], working with continuous cultures and low levels of NO_3^-, could not detect a coupling between N_2-fixation and NO_3^--respiration in *A. brasilense*. In a more recent study, Bothe *et al.* [7] demonstrated that NO_3^--respiration supported N_2-fixation only in such cells which had synthesized nitrogenase. NO_3^--dependent C_2H_2-reduction commenced approximately 1 h after the addition of NO_3^-

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and the removal of O_2 from the cultures. The reaction, however, lasted only 3–5 h when it stopped abruptly. The causes for the termination of the reaction have not been elucidated. The present investigation characterizes NO_3^--dependent N_2 fixation by *Azospirillum* spp. in more detail.

Materials and Methods

Two different strains were used: the type strain, *Azospirillum brasilense* Sp 7 (ATCC 29145), and the Cd strain, *A. brasilense* ATCC 29729. This carotenoid forming bacterium originally isolated from *Cynodon dactylon* in California [8] was kindly supplied to us by Dr. Y. Okon, Rehovot. The starter culture was grown for 1–2 d in the semisolid NFb medium [9] supplemented with NH_4Cl (1 g/l). Growth under N_2-fixing conditions was performed in the batch culture apparatus described by Volpon *et al.* [10] using the nitrogen-free liquid medium B described previously [7] and a dissolved pO_2 of 0.002–0.003 atm throughout the experiment. At the beginning, the apparatus was inoculated with the starter culture of $O.D._{560\,nm} = 1.1-1.5$ (inoculum 10% of the final volume). Growth was monitored by the absorbance at 560 nm using a spectrophotometer B 295 II model. Aliquots of the cells were taken out anaerobically at different optical densities and immediately transferred to the assay vessels.

Oxygen-dependent C_2H_2-reduction activities (Figs. 1 and 2) were assayed in 26.5 ml McCartney bottles containing 10 ml of cell suspensions ($O.D._{560\,nm} = 0.1$, 0.25 or 0.5) for 1 h at 30 °C in a shaking water bath (GFL-company, FRG, shaking rate = 50 strokes/min). The McCartney bottles were first repeatedly evacuated and flushed with nitrogen, then the cell suspensions were injected anaerobically into them, and the reaction was started by the addition of C_2H_2(2 ml) and O_2 (as indicated in the Figs.).

For measuring NO_3^--dependent C_2H_2-reduction (Figs. 3–5), N_2-grown cells of strain Cd were assayed in 125 ml Erlenmeyer flasks covered with suba seals. The vessels in the experiment of Fig. 3 contained 50 ml cell suspension ($O.D._{560\,nm} = 0.78$) and 2×10^{-2} M $NaNO_3$. The flasks were repeatedly evacuated and flushed with N_2, and the assay was started by the addition of 10 ml C_2H_2. In the experiments of Figs. 4 and 5, 10 ml of the cell suspensions ($O.D._{560\,nm} = 1.135$ for Fig. 4 and 0.78 for Fig. 5) were anaerobically transferred to dialysis bags (Kalle Company, Wiesbaden-Biebrich, FRG) which were then placed into the 125 ml Erlenmeyer flasks containing 100 ml of medium B [7] supplemented with 2×10^{-2} M $NaNO_3$. As controls, 100 ml (O.D. = 1.135, Fig. 4) or 50 ml (O.D. = 0.78, Fig. 5) cell suspensions were assayed in the 125 ml Erlenmeyer flasks. The gas phase was always N_2, and the experiments were started by the addition of 10 ml C_2H_2. The assays (Figs. 3–5) were performed at 30 °C in the shaking water bath (50 strokes/min), and every h samples were taken for C_2H_4, N_2O, CO_2, NO_2^- and NH_4^+-determinations.

Nitrite-content was measured by the naphthylethylenediamine/sulphanilamide couple and NH_4^+ by Berthelot's nitroprussiate reagent. C_2H_4-formation was followed by gas chromatography using a flame ionisation detector and a Porapak R column, and N_2O as well as CO_2 was determined in a gas chromatograph fitted with a thermal conductivity detector and a Porapak Q column with He as carrier gas. Cell-free nitrogenase extracts were prepared as described by Okon *et al.* [11]. The 14 ml assay vessels contained in a final volume of 1.35 ml : 6.25 mg crude nitrogenase extract and the following in µmol: Hepes-buffer, pH 7.3, 100; ATP, 5; creatine phosphate, 20; $MgCl_2$, 20; $MnCl_2$, 0.6; $Na_2S_2O_4$, 13.5; $NaNO_2$ and NH_4Cl as indicated; creatine phosphokinase (Sigma) 0.2 mg. Gas phase 92% N_2, 8% C_2H_2. The reaction was performed at 30 °C for 1 h under shaking.

Results

A) O_2-sensitivity of C_2H_2-reduction by Azospirillum brasilense Cd and Sp 7

To compare the O_2-sensitivity of C_2H_2-reduction of a red strain of *A. brasilense* (Cd) with that of a nonpigmented strain (Sp 7), both were grown in a fermenter under N_2-fixing batch culture conditions, and nitrogenase activity was assayed at different optical densities. Even at low concentrations of cells, the Cd culture had a reddish appearance due to the formation of carotenoids [2], whereas the Sp 7 strain was always pale brown. Fig. 1 demonstrates that the O_2-sensitivity of C_2H_2-reduction by *A. brasilense* Cd severely depended on the concentration of bacteria in the assay. A typical bell-shaped curve was obtained when the concentration of cells assayed was low ($O.D._{560\,nm} = 0.1$) because small amounts of O_2 are

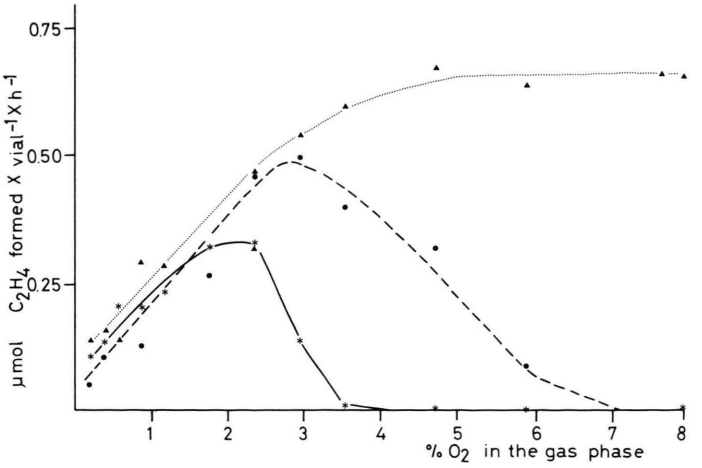

Fig. 1. Dependence of C_2H_2-reduction by *A. brasilense* Cd on the O_2-concentration in the gas phase. The cells were grown in a fermenter under N_2-fixing conditions as a batch culture and assayed for O_2-dependent C_2H_2-reduction at different optical densities (= different growth stages). For experimental conditions see Materials and Methods. Symbols: * —— * assay performed at O.D.$_{560\,nm}$ = 0.1 (in the fermenter); ● – – – ● assay performed at O.D.$_{560\,nm}$ = 0.25; ▲·····▲ assay performed at O.D.$_{560\,nm}$ = 0.5.

required to saturate respiration. At this concentration of bacteria, the optimum was at 2% O_2 in the gas phase. Higher levels of O_2 inhibited probably because they irreversibly damaged nitrogenase as these bacteria are devoid of an effective protection mechanism [12, 13]. The optimal pO_2 concentration shifted to 3–4% in the gas phase when the cell density in the vessels was increased to O.D. = 0.25. At O.D. = 0.5, C_2H_2-reduction was no longer affected by 8% O_2 in the gas phase. At even higher optical densities, the cultures performed C_2H_2-reduction in air. The overall respiratory activity of these cells lowered the O_2-tension in the cultures to concentrations which did not affect the nitrogenase.

A similar pattern of results was obtained with the nonpigmented strain Sp 7. Inhibition of C_2H_2-reduction by O_2 was obtained with low concentrations of cells in the vessels, and the optimal pO_2 in the gas phase shifted to higher concentrations with higher amounts of bacteria in the cultures. There was no consistent difference in the O_2-sensitivity of C_2H_2-reduction between the two strains in any of the many experiments performed in such a way. The same was true when frozen material of both strains was compared side by side. Similarly, a nonpigmented isolate separated from the culture sent to us by Dr. Okon (see [2]) had no different C_2H_2-reduction characteristics. The optimal O_2-concentration in the gas phase severely depended on the shaking rate of the water bath used in the test. Higher shaking rates obviously increased the level of dissolved O_2 in the culture which shifted the optimal pO_2 in the gas phase to lower concentrations. When identical conditions were employed, there was, however, never a significant difference in the O_2-optimum for C_2H_2-reduction activities between the strains used.

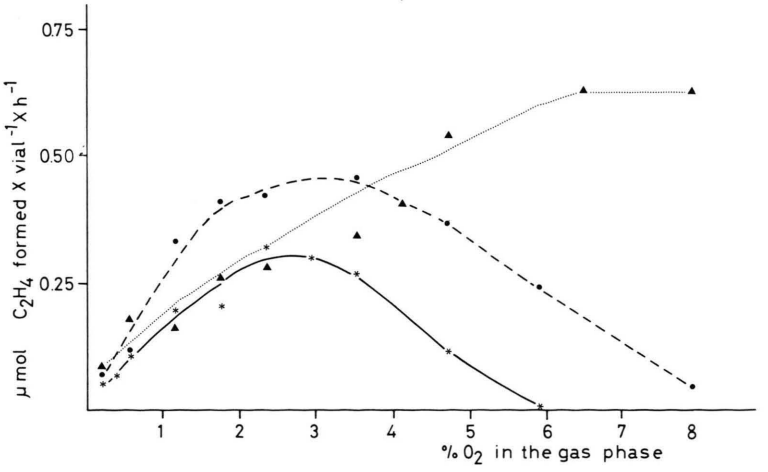

Fig. 2. Dependence of C_2H_2-reduction by *A. brasilense* Sp 7 on the O_2-concentration in the gas phase. Experimental conditions and symbols as in Fig. 1.

B) Nitrate-dependent N_2-fixation (C_2H_2-reduction)

The experiment of Fig. 3 confirms the results of the preceeding publication [7] and adds data for NH_4^+, CO_2 and N_2O formations. In this assay performed with a dense culture (O.D.$_{560\,nm}$ = 0.78), NO_3^--dependent C_2H_2-reduction abruptly stopped 3–5 h after the removal of O_2 and addition of NO_3^- as observed in similar experiments with lower concentrations of cells (O.D. = 0.1–0.5) [7]. In contrast, NO_2^- was continuously formed, and this formation paralleled with a release of CO_2 (Fig. 3). The latter of which was marginal in controls without NO_3^- indicating the limited respiratory activity due to the lack of an electron acceptor. N_2O-formation commenced 4–5 h after the addition of nitrate (Fig. 3) and thus at a time where C_2H_2-reduction had already stopped. This indicates that the NO_2^--concentration in the culture had to reach at least 2×10^{-3} M before N_2O-formation could start (see Fig. 3) and/or the synthesis of the enzyme(s) catalyzing the reduction of NO_2^- to N_2O lasted for 4–5 h. The cells excreted some NH_4^+ under the assay conditions employed (Fig. 3). The pattern of this formation was different from that of C_2H_2-reduction indicating that

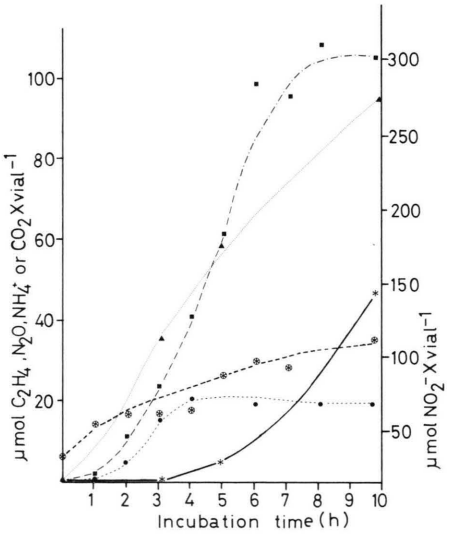

Fig. 3. NO_3^--dependent nitrogenase activity in *Azospirillum brasilense* Cd. The cells were grown in the fermenter under N_2-fixing conditions (limiting O_2-concentrations) and tested for activities at O.D.$_{560\,nm}$ = 0.782. For experimental conditions see Materials and Methods. Symbols: ▲ ····· ▲ CO_2-formation; * —— * N_2O-formation; ⊛ – – – ⊛ NH_4^+-formation; ● - - - ● NO_3^--dependent C_2H_2-reduction; ■ –·–·– ■ NO_2^--formation (note the different scale for NO_2^--formation at the right ordinate).

Table I. Inhibition of cell-free nitrogenase from *Azospirillum brasilense* Sp 7 by $NaNO_2$ and NH_4Cl.

Concentration of NO_2^- or NH_4^+ [M]	Rate	Inhibition [%]
Inhibition by $NaNO_2$:		
1. 0	164	–
2. 1.25×10^{-5}	136	17
3. 5×10^{-5}	104	37
4. 1×10^{-4}	82	50
5. 4×10^{-4}	70	57
Inhibition by NH_4Cl:		
1. 0	140	–
2. 1×10^{-3}	60	58
3. 2×10^{-3}	50	64

The rate is given in nmol C_2H_4 formed/h×mg protein. For the preparation of the cell-free nitrogenase and the assay conditions see Materials and Methods.

it was due to NO_3^--respiration and not to N_2-fixation. It has not yet been elucidated whether NH_4^+-formation is catalyzed by an enzyme system different from that catalyzing N_2O-formation as in *Citrobacter* sp. [14].

NO_2^- and/or NH_4^+ could have been the factors which stopped NO_3^--dependent C_2H_2-reduction after 3–5 h. NO_2^- is known to block the molybdenum-iron part of nitrogenase as described for the *Rhizobium* enzyme [15], and NH_4^+ may affect the regulatory properties of the nitrogen fixing system of *Azospirillum* [10]. The data presented in Table I, indeed, indicate that NO_2^- and NH_4^+ inhibit cell-free nitrogenase activity of *A. brasilense*. $Na_2S_2O_4$- and ATP-dependent C_2H_2-reduction by cell-free extracts was inhibited to 50% by approximately 1×10^{-4} M $NaNO_2$ and 1×10^{-3} M NH_4Cl.

To decide whether NO_2^- and/or NH_4^+ were, indeed, the crucial factors blocking NO_3^--dependent C_2H_2-reduction, an experiment was performed where these substances were dialysed away from the cells. For this, 10 ml of N_2-fixing *A. brasilense* Cd were assayed in a dialysis bag which was placed into 100 ml culture medium. At the end of the experiment (after 8 h), the concentration of NO_2^- in the dialysis fluid was 2.02×10^{-3} M and in the dialysis bags (supernatant after centrifugation) 3×10^{-3} M indicating that 87% of the NO_2^- had appeared in the dialysis fluid (Fig. 4). The concentration of NH_4^+ was negligible in this experiment (not documented). Nitrate dependent C_2H_2-reduction stopped after 5 h when the NO_2^--content in the dialysis fluid had reached 10^{-3} M. The reaction was terminated after exactly the

same time in control cultures in which the NO_2^--content was not lowered by dialysation. Such controls were a 100 ml culture of the same batch, the same optical density, the same Erlenmeyer flasks and also otherwise identical conditions (Fig. 4) or 10 ml in the conventional 26.5 ml McCartney bottles (not documented). In the former, the amount of NO_2^- in the medium steadily increased during the first 4 h of the experiment up to 4.5×10^{-3} M and then decreased due to the onset of NO_2^--dissimilation. Fig. 5 shows a similar experiment using lower concentrations of cells in the assay. In this case C_2H_2-reduction also stopped 3–5 h after the addition of NO_3^- and ir-

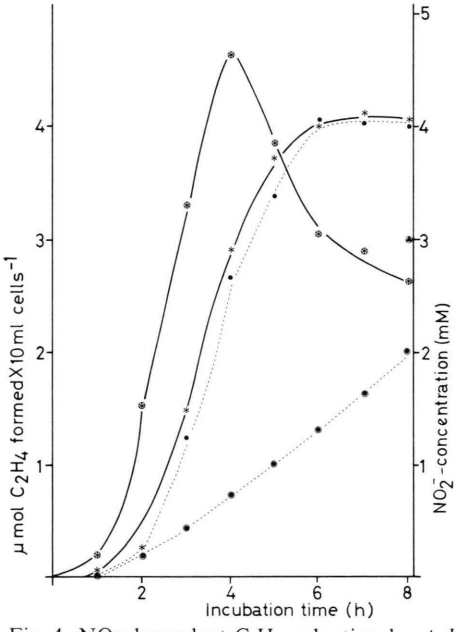

Fig. 4. NO_3^--dependent C_2H_2-reduction by *A. brasilense* Cd in dialysis bags where the NO_2^- and NH_4^+ formed were continuously diluted out. Assay performed at high concentrations of cells ($O.D._{560\,nm}$ = 1.135; late log. phase) *A. brasilense* Cd was grown in the fermenter under N_2-fixing conditions and tested at O.D. = 1.135. A) * —— * C_2H_2-reduction, assay performed in 125 ml Erlenmeyer flasks containing 100 ml cell suspension, 2×10^{-2} M $NaNO_3$ under N_2/C_2H_2 (see Materials and Methods). For comparison with B, the nitrogenase activity is expressed per 10 ml cell suspension. ⊛ —— ⊛ NO_2^--concentration (in mM). B) ● - - - ● C_2H_2-reduction, assay performed in 125 ml Erlenmeyer flasks containing 100 ml of NO_3^--medium (2×10^{-2} M $NaNO_3$) and a dialysis bag with 10 ml *A. brasilense* Cd. The nitrite and ammonia was dialysed out of the bag (at the end 87% of the nitrite formed had been dialysed out) whereas the bacteria remained in it. Gas phase N_2/C_2H_2. ⊙ - - - ⊙ NO_2^--content in the dialysis fluid; ▲ NO_2^--content in the dialysis bag at the end of the experiment. The content was determined by taking the cells out of the bag, centrifuging and assaying the supernatant.

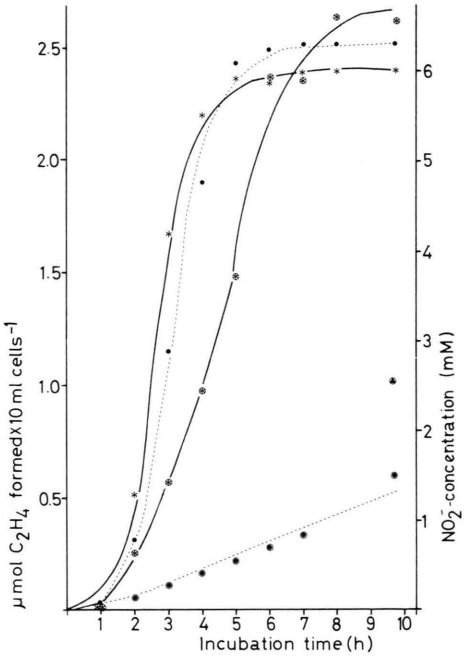

Fig. 5. NO_3^--dependent C_2H_2-reduction by *A. brasilense* Cd in dialysis bags where the NO_2^- and NH_4^+ formed were continuously diluted out. Assay performed at lower concentrations of cells ($O.D._{560\,nm}$ = 0.78; middle log. phase). Same experimental conditions as in Fig. 4 except that 50 ml cell suspension was used in the control experiment.

respectively of the NO_2^--concentration in the medium. All these results indicate that NO_2^- and NH_4^+ are unlikely the primary factors causing the termination of NO_3^--dependent C_2H_2-reduction in the intact cells.

Discussion

Nur *et al.* [2] suggested that carotenoids of *Azospirillum brasilense* Cd play a role in the protection of the sensitive nitrogenase system from damage by oxygen. These authors also observed a clear relationship between the carotenoid content in *A. brasilense* Cd and the O_2-content in the growth medium [16]. The present communication, however, indicates that *A. brasilense*, though forming carotenoids, does not tolerate higher levels of O_2 than strain Sp 7 in nitrogen fixation (C_2H_2-reduction). Difficulties also have to be encountered to explain the mechanism by which carotenoids could protect nitrogenase. In photosynthesis, carotenoids are known to function against photooxidative damage [17]. The chlorophyll molecule may irreversibly be destroyed by O_2 in the

excited triplet state, obtained by illumination with high light intensities. Strong light may also cause a photoreaction of other molecules with O_2 and the generation of superoxide and peroxide radicals which may destroy structures unspecifically. Carotenoids can protect these molecules by dissipating excess of light quanta from photolabile compounds. It has been demonstrated that photosynthesis is more resistent to illumination by strong light in such organisms which synthesize carotenoids than in those without these isoprenoids [18, 19].

Protection of nitrogenase from damage by O_2, however, requires a quantitative removal of O_2 from the nitrogenase site, in the dark. It is difficult to conceive how carotenoids can fulfil such a function. They would have to react directly with O_2 or form a barrier against the free diffusion of the gas. Both of which is unlikely. Carotenoids may, however, be beneficial to such *Azospirillum* strains that live at or near the surface of the soil and are exposed to strong light. *Azospirillum* lives in association with roots of grasses where it has not to cope with a light problem. It is not known whether *Azospirillum* Cd and other carotenoid-forming strains occur at the surface of soils.

The present communication characterizes NO_3^--dependent N_2-fixation (C_2H_2-reduction) in more detail than the preceeding one [7]. The factors have now been elucidated which cause the reaction to come to a halt after 3–5 h. The termination was not due to exhaustion of NO_3^- in the medium, as suggested by Scott *et al.* [4], because the addition of fresh NO_3^- to the cells did not reactivate C_2H_2-reduction [7]. Several observations indicate that the end of nitrate-dependent C_2H_2-reduction was not caused by inhibition by NO_2^- or NH_4^+: The duration of the reaction was not extended by lowering the content of NO_2^- in the cultures by dialysation. NO_3^--dependent C_2H_2-reduction always proceeded only 3–5 h after the addition of NO_3^-, irrespectively of whether high or low concentrations of cells were assayed in the vessels (comp. [4] and Figs. 4 and 5 and data in [7]). In the experiment of Fig. 5, the concentration of NO_2^- was 4×10^{-4} M after 4 h when the reaction ceased, and C_2H_2-reduction by cell-free nitrogenase proceeded with 43% of the maximal rate at such NO_2^--concentration (Table I). Generally, about ten fold higher concentrations of an inhibitor are required to block an enzyme reaction in intact cells as compared to cell-free conditions. Previous work had shown that NO_3^--dependent nitrogenase activity can proceed at NO_2^--concentrations up to 5×10^{-3} M under different experimental conditions [3, 4]. As pointed out previously [3, 4], *Azospirillum* possesses a mechanism to exclude deleterious amounts of NO_2^-. This is probably achieved by an active NO_2^- excretion process. The amount of NH_4^+ formed was smaller than that of NO_2^-, and the concentration of NH_4^+ which affected cell-free nitrogenase was even higher than that of NO_2^- (Table I). Thus other factors besides NH_4^+ and NO_2^- must be responsible for the termination of NO_3^--dependent C_2H_2-reduction. Previous experiments [7] had shown that the synthesis of the enzymes of assimilatory nitrate reduction (determined by the formation of nitrite reductase) takes 3–5 h and that this coincides with the duration of NO_3^--dependent C_2H_2-reduction, whereas this latter reaction is expressed within 1 h [4, 7]. All these observations lead to the conclusion that the cells perform NO_3^--dependent N_2-fixation only until the assimilatory NO_3^--reduction pathway is operating. *Azospirillum* performs NO_3^--dependent N_2-fixation as a transitory reaction, and then gives up N_2-fixation in favour of assimilatory nitrate reduction, as this reaction is possibly more energy saving. Thus NO_3^--dependent N_2-fixation is physiologically an interesting reaction, but it is probably of limited value for the organisms in the field, possibly when sudden changes of the environment demand a massive supply of N_2-fixation products for the synthesis of new enzymes.

Acknowledgements

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