Nitrite Inhibition of Bacterial Dinitrogen Fixation

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Z. Naturforsch. **37 c**, 784–786 (1982); received March 16/May 26, 1982

Bacterial Nitrogenase, Nitrite Inhibition, Dinitrogenase Reductase

Dinitrogen fixation by crude extracts from *Rhodopseudomonas palustris* and *Rhodomicrobium vannielii* and by purified nitrogenase preparations from *Azotobacter vinelandii* and *Clostridium pasteurianum* is inhibited by nitrite, whereas in the same preparations nitrate is without effect. In *Clostridium* nitrite seems to interact with dinitrogenase reductase component of the bacterial nitrogenase complex.

Introduction

It is widely recognized that amonia and other forms of reduced nitrogen cause a strong inhibition of dinitrogen fixation by intact cells of various microorganisms, especially phototrophic bacteria [1, 2]. Nitrite inhibits *in vivo* acetylene reduction by *R. capsulata* [3]. Likewise, nitrite has been also described as a potent inhibitor of nitrogenase activity in *Rhizobium* bacteroids extracted from nodules [4], in N_2-fixing cultures of rhizobia [5] and of rhizobial nitrogenase *in vitro* [6]. Very recently, inhibition of purified dinitrogenase from *Clostridium pasteurianum* by nitrite and NO has been also reported [7].

In this communication the inhibition by nitrite of acetylene reduction in crude extracts from *R. palustris* and *R. vannielii* as well as in purified nitrogenase preparations from *A. vinelandii* and *C. pasteurianum* is presented. In this latter bacterium the effect of nitrite seems to be exerted by reacting preferentially with the dinitrogenase reductase component of the whole nitrogenase complex.

Materials and Methods

Cell cultures

R. palustris (ATCC 17001) has grown photoheterotrophically with succinate and N_2 as previously described [1].

R. vannielii strain 166 was grown in a medium containing per liter: KH_2PO_4, 0.5 g; $MgSO_4$, 0.4 g; NaCl, 0.4 g; $CaCl_2 \cdot 2H_2O$, 0.05 g; disodium succinate, 2 g; yeast extract, 0.5 g; ethanol, 0.5 g; ferric citrate, 0.005 g, and trace solution as described for *R. palustris* [1], 1 ml.

C. pasteurianum W5 and *A. vinelandii* OP were grown as described elsewhere [8, 9].

Cell-free extracts

Cells were collected anaerobically by centrifugation at $10000 \times g$ for 15 min, washed once with 50 mM Tris-HCl buffer (pH 8.0), and resuspended in the same buffer containing 1 mM sodium dithionite. Cells were broken by sonication and crude extracts were obtained as previously described [1].

Nitrogenase components from *C. pasteurianum* and *A. vinelandii* were isolated and purified by previously described methods [8, 9].

Nitrogenase assays

Dinitrogen fixation was measured by the acetylene reduction technique. The reaction mixtures were prepared according to Zumft and Castillo [1].

Analytical Methods

Protein was estimated according to the Lowry procedure [10] using bovine serum albumin as standard. Nitrite was measured as described by Snell and Snell [11].

Results and Discussion

Ammonium salts, glutamine, asparagine and urea cause an immediate and reversible inactivation of

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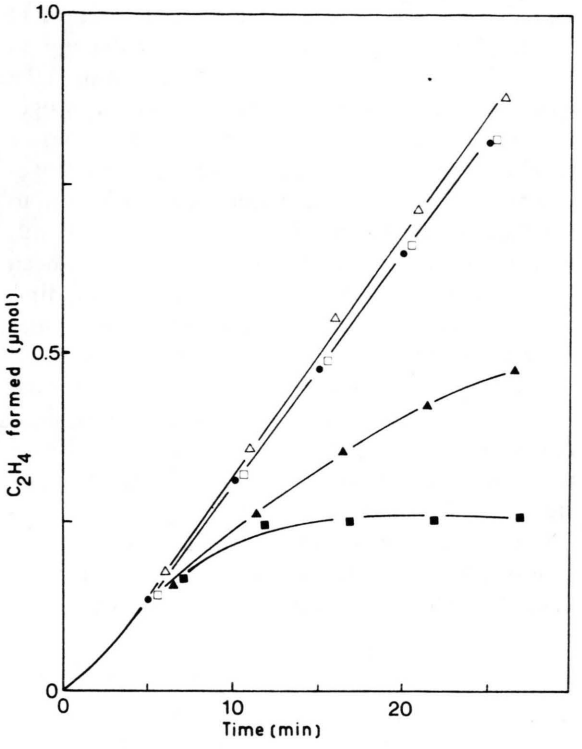

Fig. 1. Inhibition by nitrite of dinitrogenase from *R. vannielii*. The assays were performed with reaction mixtures containing extracts from *R. vannielii* (2.25 mg protein) and the following additions: none (●–●); 1 mM KNO_3 (□–□); 2 mM KNO_3 (△–△); 1 mM KNO_2 (▲–▲); 2 mM KNO_2 (■–■).

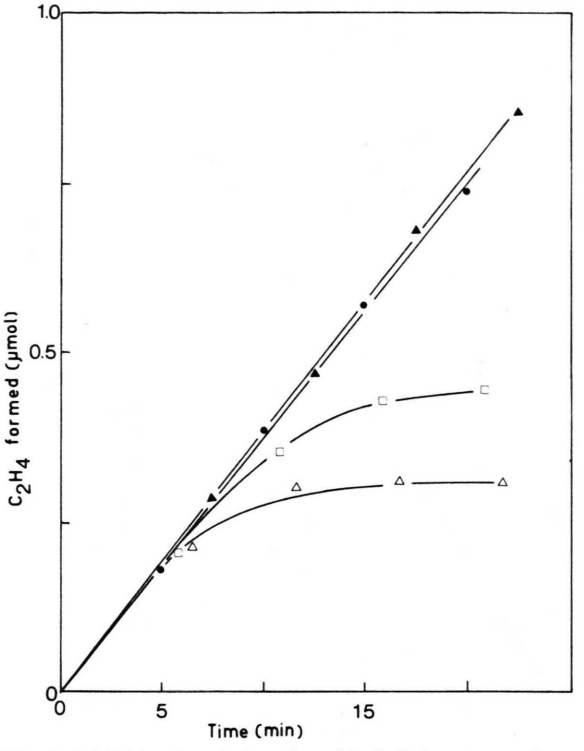

Fig. 2. Inhibition by nitrite of purified dinitrogenase from *A. vinelandii*. The assays were carried out with partially purified Fe protein (0.1 mg) and Mo Fe protein (0.2 mg) from *A. vinelandii* in the absence (●–●) or in the presence of 1 mM KNO_3 (▲–▲); 1 mM KNO_2 (□–□) and 2 mM KNO_2 (△–△).

light-dependent acetylene reduction in intact cells of phototrophic bacteria [1, 2].

Addition of nitrite to crude extracts from *R. vannielii* (Fig. 1) and *R. palustris* (results not shown) rapidly suppresses acetylene reduction. A similar inhibition by nitrite has been reported for nitrogenase of alfalfa root nodules [12], in N_2-fixing cultures of rhizobia [5] and for nitrogenase from crude extracts of *Rhizobium japonicum* [6] where a reversible competitive inhibition that can be reversed by gel filtration has been also described (13).

Partially purified nitrogenases from *A. vinelandii* and *C. pasteurianum* are also inhibited by nitrite (Fig. 2 and 3). In *Clostridium* inhibition seems to affect the dinitrogenase reductase component of the nitrogenase complex since addition of Fe protein to inhibited enzyme relieved the inhibition whereas addition of Mo Fe protein did not (Fig. 3). In a separate experiment addition of Mo Fe protein to a reaction mixture containing nitrogenase previously inactivated by nitrite did not overcome the inhibition (Results not shown). These results are at variance with the reported nitrogenase inhibition by nitrite in *Rhizobium* where binding of the inhibitor to the Mo Fe protein component has been proposed [13]. By contrast, an irreversible inhibition of clostridial nitrogenase by nitrite due to binding of the inhibitor to the Fe protein has been recently reported [7]. Apparently nitrite inhibits nitrogenase by being reduced to NO which irreversibly and rapidly inactivates the Fe protein while the Mo Fe protein remains undamaged [7].

Addition of nitrate either to crude extracts from phototrophic bacteria or to purified enzyme preparations from *A. vinelandii* and *C. pasteurianum* does not affect dinitrogenase activity (Figs. 1–3). This lack of inhibition can be attributed to the absence of nitrate reductase activity from these preparations.

The nitrate-induced depression of rhizobial dinitrogenase has be explained by assuming that nitrite

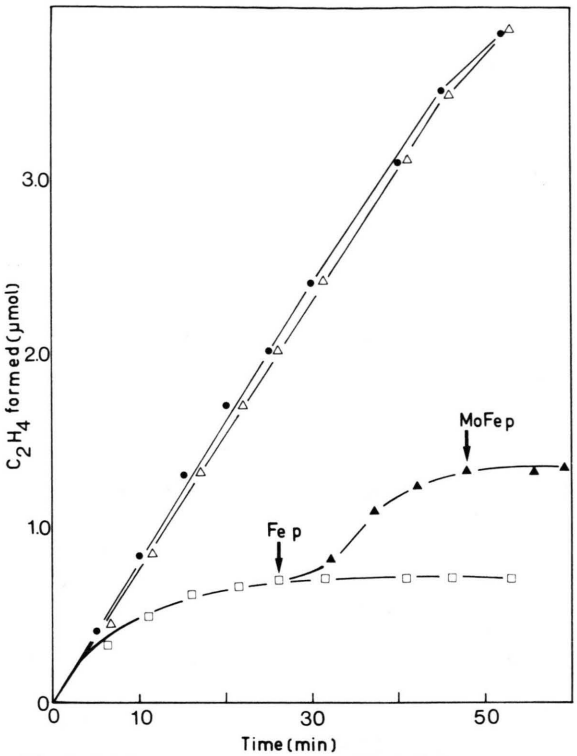

Fig. 3. Inhibition by nitrite of purified dinitrogenase from *C. pasteurianium*. The assays were performed with partially purified Fe protein (0.05 mg) and Mo Fe protein (0.15 mg) from *C. pasteurianum* in the absence (●–●) or in the presence of 2 mM KNO_3 (△–△) and 1 mM KNO_2 (□–□). At the times indicated by the arrows, 0.05 mg Fe protein (▲–▲) and 0.15 mg Mo Fe protein (▲–▲) were added to the reaction mixture containing KNO_2.

is produced in nodules by nitrate reductase present therein [14]. The presence of nitrate reductase in nodules lacking nitrite reductase [15] determined the appearance of nitrite which can inactivate dinitrogenase. Additional and/or alternative explanations of the effect of nitrate and nitrite on rhizobial nitrogenase by preventing the inactivated leghemoglobin to penetrate into bacteroids [16] or by depressing nodulation [17] or leghemoglobin synthesis [18] have been also suggested. By contrast, a stimulation of dinitrogenase activity in the presence of nitrate under anaerobic conditions have been reported in *Spirillum lipoferum*. Apparently the bacterium dissimilates nitrate to obtain the ATP required for dinitrogen reduction and excretes to the medium the nitrite formed in the process, thus avoiding the presence of inhibitor inside the cells [19].

The lack of nitrate inhibition in crude extracts from phototrophic bacteria clearly indicates the absence of nitrate reductase in the organisms studied.

Acknowledgements

Part of this work was done in the Botanisches Institut der Universität Erlangen-Nürnberg. The authors thank Prof. Dr. E. Kessler and Prof. Dr. W. G. Zumft for hospitality and helpful advices. One of us (F.C.) acknowledges the financial support of the Krupp Foundation.

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