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EFFECTS OF IN VIVO TREATMENTS ON THE ACTIVITY OF NITROGENASE ISOLATED FROM *RHODOSPIRILLUM RUBRUM*

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Nitrogenase activities of partially purified extracts of *Rhodospirillum rubrum* grown on different nitrogen sources were examined. Most of the nitrogenase from cells grown on N_2 or glutamate was in the inactive form. This form was also predominant in extracts from cells grown on limiting N_2 or glutamate plus N_2. The enzyme from cells grown with limiting NH_4^+ was fully active. Nitrogenase displayed varying degrees of sensitivity to in vivo inhibition by NH_4^+, depending on the culture conditions. However, addition of NH_4^+ to the cultures prior to harvest did not change the proportion of the active form of the enzyme in extracts from that found in control samples. Several of these observations are inconsistent with the three component model of nitrogenase regulation of Yoch and Cantu (Yoch, D.C. and Cantu, M. (1980) J. Bacteriol, 142, 899–907). A regulatory system controlled by products of NH_4^+ assimilation is suggested.

Introduction

Nitrogenase activity in purple nonsulfur bacteria can be inhibited rapidly and reversibly by NH_4^+ under certain conditions. This effect has been observed only in vivo, and it probably is mediated by products of NH_4^+ assimilation rather than by NH_4^+ directly. There is reason to believe that this phenomenon is not inhibition in the classic sense; loss of activity may be due to reversible inactivation of the enzyme.

Depending on the source of nitrogen used to grow the cells, nitrogenase iron protein can be isolated in either the active or inactive form [1,2]. In *Rhodospirillum rubrum*, the inactive form can be activated in the presence of Mn^{2+} and MgATP by a component isolated from chromatophores. This component, designated activating enzyme, appears to catalyze the release of a small, covalently bound molecule from the nitrogenase iron protein during the activation process [3]. In vitro inactivation of the *R. rubrum* iron protein has not been reported.

Carithers et al. [2] and Yoch and Cantu [4] developed a model that describes the *R. rubrum* system in terms of three interconvertible forms of the enzyme. They suggested that nitrogenase A is always active, while nitrogenase R can be active or inactive, depending on specific conditions. The results of our initial study of in vivo responses of *R. rubrum* to NH_4^+ were not in total agreement with this hypothesis [5]. This report extends these findings and describes the effects of in vivo treatments on the activity of isolated nitrogenase.

Methods

Culture conditions

R. rubrum (a derivative of ATCC 11170) was

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Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; MSX, DL-methionine-(±)-sulfoximine.

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grown photosynthetically in 500 ml polycarbonate centrifuge bottles on the malate medium of Ormerod et al. [6] as modified by Sweet and Burris [5]. The source of nitrogen was either 5 mM glutamate, 0.5–1.5 mM NH_4Cl, N_2 bubbled gently through the medium (10 ml per min), or N_2 above the medium without bubbling (limiting N_2), as specified. When sparged or limiting N_2 was used, 0.5–1.0 mM NH_4Cl was provided initially, but this was exhausted at least 5 h before cells were used for experiments. The centrifuge bottle lids were modified to hold a vaccine stopper and an O-ring, so that the proper anaerobic gas phases could be maintained (Ar or N_2), and the cells could be harvested without transfer to another vessel. Other details of culture conditions and measurement of cell density have been reported [5].

Preparation of extracts

Cells were harvested by centrifugation at 1000 $\times g$ for 10 min, and the supernatant fluid was discarded. This and subsequent steps were performed anaerobically with Ar as the gas phase. The sedimented cells were resuspended in 2–3 ml of 50 mM Hepes (pH 8.0) containing 30 mM EDTA, 2 mM $Na_2S_2O_4$, and 5 mg of lysozyme per ml. This mixture was kept at room temperature for 15 min with occasional agitation. The pH was maintained above 7.0 with the dropwise addition of 1 M K_2CO_3 as necessary. The suspension then was injected through a hypodermic needle into 4–5-times its volume of dilute buffer (0.5 mM Hepes/1 mM $Na_2S_2O_4$, pH 8.0) with vigorous mixing. $MgCl_2$ and deoxyribonuclease were added to final concentrations of 10 mM and 0.1 mg per ml, respectively. This suspension was kept at room temperature for 10 min with occasional stirring, and it then was centrifuged at 30000 $\times g$ for 30 min at 5°C.

The clear supernatant solution was passed through a DEAE-cellulose column (1 × 6 cm) that had been equilibrated with 20 mM Tris-HCl buffer (pH 7.4)/50 mM NaCl/1 mM $Na_2S_2O_4$. The column was washed with approx. 2 bed volumes of 20 mM Tris-HCl/0.1 mM NaCl; this eluted activating enzyme but the nitrogenase proteins were retained. The brown band containing nitrogenase was eluted with 20 mM Tris-HCl/0.4 M NaCl. This preparation was used in the assays described subsequently.

Activating enzyme was obtained from cells grown on glutamate in larger batches. After a lysis procedure similar to that described, the material sedimented at 30000 $\times g$ was resuspended in 20 mM Tris-HCl buffer (pH 7.4)/0.5 M NaCl/1 mM $Na_2S_2O_4$/2 mM dithiothreitol. This suspension was centrifuged at 30000 $\times g$ for 1 h, and the supernatant solution was used as the source of activating enzyme.

Enzyme assays

Nitrogenase was assayed by its evolution of H_2 at 25°C as measured with a H_2 electrode [5,7]. Samples of a culture were transferred anaerobically into the electrode chamber. The reaction mixture for cell-free preparations contained 50 mM Hepes (pH 7.5), 5 mM ATP, 30 mM creatine phosphate, 0.5 mM $MnCl_2$, 25 mM $MgCl_2$, 5 mM $Na_2S_2O_4$ and 0.05 mg creatine phosphokinase per ml. Solutions were made anaerobic by sparging with Ar, so that H_2 evolution provided a measure of total nitrogenase activity (no N_2 present to serve as an electron acceptor). Rates were measured by drawing tangents to the H_2 electrode traces at $T = 30$ min. The activity at 30 min in the presence of activating enzyme was taken as 100%, and the amount of the active form of the enzyme was calculated by comparing this rate to the activity in the absence of activating enzyme. When very low rates were obtained (i.e., inactive enzyme in the absence of activating enzyme), larger amounts of extract were used to minimize the dilution effect [8].

Protein was precipitated with trichloroacetic acid and its concentration was measured by the microbiuret method of Goa [9]; bovine serum albumin served as a standard. Sources of chemicals have been recorded earlier [5,10].

Results and Discussion

Nitrogenase activity of intact cells

Sweet and Burris [5] discussed the effects of NH_4^+ and DL-methionine-(±)-sulfoximine (MSX) on H_2 evolution by intact cells under four conditions of nitrogen availability. Fig. 1 shows these effects for a fifth condition: cells grown with limiting N_2. When MSX was added first (upper trace),

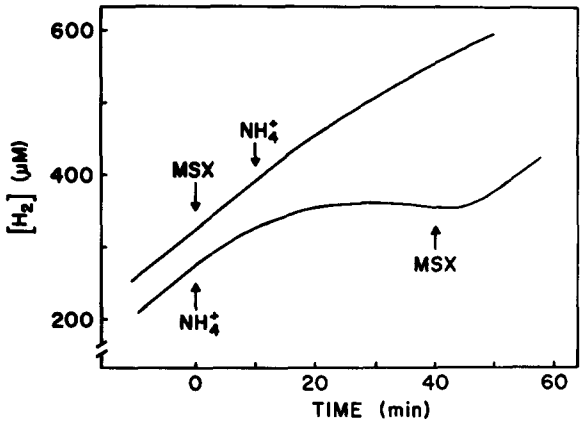

Fig. 1. H_2 evolution by intact cells of *R. rubrum* grown with limiting N_2. NH_4Cl was added to 0.2 mM and MSX was added to 0.05 mM, final concentrations, where indicated. Cell density: $A_{600} = 0.42$.

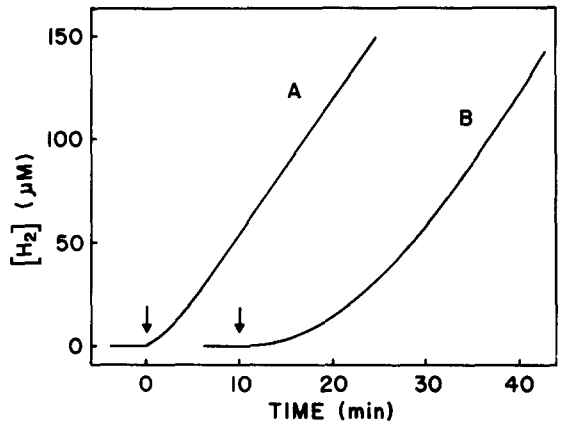

Fig. 2. H_2 evolution by partially purified nitrogenase from *R. rubrum*. The reaction was started by addition of the enzyme at the arrows. Trace A shows enzyme from NH_4^+-limited cells at a protein concentration of 0.062 mg per ml, with no activating enzyme added. Trace B (offset for clarity) shows enzyme from cells grown on glutamate at a protein concentration of 0.17 mg per ml, with activating enzyme added (an additional 0.013 mg protein per ml). Other assay conditions are given in the text.

NH_4^+ produced very little effect. When NH_4^+ was added first (lower trace), there was a gradual inhibition of H_2 evolution, and inhibition was 50% at about 20 min. The subsequent addition of MSX reversed this inhibition. Thus, the in vivo effect of NH_4^+ on these cells was intermediate between the extremes seen in the previous study. Nitrogenase activity in cells grown with N_2 or glutamate plus N_2 was sensitive to NH_4^+ (greater than 90% inhibition after 20 min); activity in cells grown with limiting NH_4^+ or glutamate only was insensitive to NH_4^+ (less than 10% inhibition after 20 min). The term 'inhibition' is used in describing these in vivo effects; however, decreased activity may be produced by a reversible inactivation process.

Activity of partially purified nitrogenase

Fig. 2 shows typical H_2 evolution from cell-free nitrogenase preparations. The active form of the enzyme (trace A) produced a linear rate after 4–5 min; the trace was identical in the presence of added activating enzyme. The reason for initial nonlinearity is not apparent; highly purified *Azotobacter vinelandii* nitrogenase with an equimolar component ratio gave a similar lag (Guth, J.H., unpublished data). The inactive form of the enzyme displayed very little activity without the addition of activating enzyme. In the presence of activating enzyme (trace B), gradual activation occurred during the measurement.

Table I summarizes the results of similar measurements of H_2 production by partially purified extracts from cells grown on various sources of nitrogen. The percentages of active nitrogenase are approximate, because the effective ratio of nitrogenase components in the extracts is not known, and it changes with time during activation.

Nitrogenase from cells grown on N_2 or glutamate was almost all in the inactive form. We presume this is because it was inactivated during harvest. Inactivation also was substantial in cells grown on limiting N_2 or glutamate plus N_2, but no inactivation occurred in cells grown on limiting NH_4^+. Addition of NH_4^+ to the medium 20 min prior to harvest had very little effect under any of these conditions (Table I). MSX added just before harvest provided slight protection against inactivation in cells from N_2 or limiting N_2 conditions; this effect was less than expected, considering that MSX produced total reversal of NH_4^+ inhibition in vivo (Ref. 5, and Fig. 1 of this work).

Several of these observations agree with earlier reports, but there is one major exception. As stated in the introduction, Yoch and Cantu [4] proposed a model that describes the regulation of nitrogenase activity in *R. rubrum* in terms of three forms of the enzyme. They reported that when NH_4^+ was added to a culture of nitrogen-starved cells, in vivo

TABLE I

EFFECTS OF IN VIVO TREATMENTS ON NITROGENASE ACTIVITY OF PARTIALLY PURIFIED EXTRACTS FROM *R. RUBRUM*

In vivo treatments were: 0.2 mM (final concentration) NH_4Cl, 20 min before harvest; 0.05 mM (final concentration) MSX, 10 min before harvest or NH_4Cl addition. Degree of sensitivity is defined in the text. Variability in the data is indicated as the range of the values, with the number of independent cultures tested in parentheses.

Nitrogen growth source	Sensitivity to NH_4^+ in vivo	Treatment in vivo	Active nitrogenase in extracts (%)
N_2	sensitive	—	<5 (2)
		NH_4^+	<5 (2)
		MSX	35±16 (3)
		MSX+NH_4^+	30±15 (2)
Limiting NH_4^+	insensitive	—	100± 2 (3)
		NH_4^+	99± 2 (3)
Limiting N_2	partially sensitive	—	31±16 (5)
		NH_4^+	29±11 (2)
		MSX	46±10 (3)
Glutamate+N_2	sensitive	—	25± 2 (2)
		NH_4^+	18± 3 (2)
Glutamate only	insensitive	—	7± 3 (2)
		NH_4^+	7± 2 (2)

nitrogenase activity was rapidly inhibited and the A form of the enzyme was converted to the inactive R form. We reported previously that in our NH_4^+-limited cultures there was no rapid in vivo inhibition of nitrogenase activity by added NH_4^+ [5]. Data presented here show that the enzyme isolated from our NH_4^+-limited cultures was fully active, even if the cells had been treated with NH_4^+ prior to harvest. Both of these observations disagree with the data and the model of Yoch and Cantu.

Our earlier results indicated that the continued presence of N_2 is important for rapid in vivo inactivation by NH_4^+. One possible reason for the discrepancy between our results and those of Yoch and Cantu is that their NH_4^+-limited cultures may have been exposed to small amounts of N_2. Our experiment with N_2-limited cells was designed to test this possibility; however, we observed only slow inhibition of nitrogenase activity by NH_4^+ under an atmosphere of 100% N_2 (Fig. 1). Furthermore, treatment of the cells with NH_4^+ prior to harvest produced the same proportion of inactive enzyme in extracts as that found in the control samples without added NH_4^+. This was observed at several cell densities, from $A_{600} = 0.39$–0.86. We conclude that small amounts of contaminating N_2 probably would not cause the degree of inhibition seen by Yoch and Cantu. Other possible reasons for the discrepancy in results include: (1) differences in the cell density (which would affect light availability); (2) differences in handling of the cells during harvest; or (3) intrinsic strain differences. We have not explored these possibilities.

Until discrepancies in results between laboratories are resolved, we remain reluctant to adopt the three component model of nitrogenase regulation. Our results support the idea that nitrogenase can be simply active or inactive, and phenomena of regulation can be explained on the basis of inhibition by NH_4^+ or a product of its metabolism [5,11]. We speculate that the presence or activity of this mechanism, in turn, may depend on a recent flux of NH_4^+ through the assimilation pathway. Data in Table I show that in the absence of glutamate, the availability of N_2 and the sensitivity of nitrogenase to in vivo inhibition by NH_4^+ correlate well with the activity of the extracted enzyme. In the presence of glutamate, the availability of N_2

still correlates with sensitivity to in vivo inhibition. However, extracts from cultures grown on glutamate always are predominately inactive, regardless of the availability of N_2 or whether NH_4^+ was added prior to harvest. These observations suggest that glutamate also has a profound effect on the regulation of nitrogenase activity. NH_4^+ assimilation appears to be important in this complex regulatory process, and some investigators have proposed that glutamine synthetase is involved directly in the *Rhodopseudomonas capsulata* system [12,13]. This also may apply to *R. rubrum*, but much more information will be required to elucidate the nature of these control mechanisms.

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