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Nitrite and Nitric Oxide as Inhibitors of Nitrogenase from Soybean Bacteroids

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Nitrite was able to strongly inhibit C_2H_2 reduction by nitrogenase from soybean bacteroids, whereas H_2 evolution was unaffected under the same conditions. NO inhibited both C_2H_2 reduction and H_2 evolution; during C_2H_2 reduction, sensitivity of nitrogenase to NO was higher than to NO_2^-, and the K_i values were, respectively, 0.056 and 0.52 mM. Production of NO resulting from a reduction of NO_2^- by dithionite in nitrogenase incubations was observed. However, the characteristics of inhibitions and the low level of NO generated by nitrite reduction ruled out the suggestion concerning a direct role of NO to explain the inhibitory effect of NO_2^- on nitrogenase.

The reduction of nitrate by nitrate reductase, a ubiquist enzyme present in plants and in most microorganisms, generates nitrite, a very reactive anion. It has been described as responsible for a strong inhibition of nitrogen fixation by isolated bacteroids from soybean nodules (9). This effect was due to an inhibition of nitrogenase itself observed with crude extracts (5) and characterized with the purified enzyme, fractionated or not (16). On the other hand, nitrite was able to react also with leghemoglobin, giving the ferric form of this hemoprotein, which is unable to carry oxygen to the bacteroids (11). The possibility for nitrite to contribute to the depressive effect of nitrate on legume nitrogen fixation was proposed and could explain this well-known phenomenon (8). The origin and role of nitrite in the nodules were questioned (3) since nitrate reductase-deficient mutants of *Rhizobium* induced the same level of nitrogen fixation inhibition as the wild type. The possibility for nodule cytosol nitrate reductase to play a role in the genesis of nitrite was suggested in *Pisum* (2) and recently confirmed in soybean (J. G. Streeter, Plant Physiol., in press) with *Rhizobium* lacking nitrate reductase.

At the same time, although nitrite was considered as a natural anion able to act on nitrogen fixation, a recent paper (6) suggested that nitrogenase inhibition may be due, not to nitrite, but rather to nitric oxide (NO), its reduction product generated in the incubations.

In this paper, we report comparative effects of nitrite and NO on purified bacteroid nitrogenase during the reduction of C_2H_2 and protons. The possibility to produce NO by nitrite reduction and its involvement in nitrogenase inhibition were investigated and discussed.

MATERIALS AND METHODS

Production of nodules and preparation of bacteroids. Soybeans (*Glycine max* Merr. cv. Altona) inoculated with *Rhizobium japonicum* strain 1809 were grown in a glass house as previously described (10), and nodules were collected about 35 days after sowing (nodule age, 25 to 26 days).

Bacteroids were prepared anaerobically from 80 g (fresh weight) of nodules as described elsewhere (15), and 600 g of nodules was generally required to perform complete nitrogenase purification.

Nitrogenase purification. Bacteroids were resuspended in 0.1 M *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES) buffer, pH 8.5, containing 1.2 mM $Na_2S_2O_4$, and disrupted in a precooled Aminco French pressure cell at $110{,}316 \times 10^6$ N m^{-2}.

The general procedure, described by Israel et al. (4) and slightly modified as previously reported (16), was routinely used to prepare purified unfractionated nitrogenase (30 to 35 mg of protein per ml). These enzyme preparations exhibited a specific activity of 100 to 150 nmol of C_2H_4 formed per min and per mg of protein.

Nitrogenase assays. The composition of incubations, prepared in duplicate, and the experimental conditions were as described previously (16). Reactions were initiated by the addition of $Na_2S_2O_4$ (10 mM) or nitrogenase extracts. C_2H_2 reduction was determined in rubber-cap vials (24 ml) filled with argon and receiving C_2H_2 to give final concentrations in the range of 0.025 to 0.2 mM. Ethylene produced was measured with a flame ionization gas chromatograph under conditions described elsewhere (5).

Assays for the measurement of H_2 evolution were conducted under the same conditions except that the gas phase was argon. H_2 was determined by analyzing 500-μl samples of gas from the reaction vessels in a gas chromatograph (Intersmat IGC 120) equipped with a thermal conductivity detector. A column (2.5 by 0.0032 m) of a molecular sieve of 0.5 nm (80 to 100 mesh) was used with argon as a carrier at a flow rate of 16 ml min^{-1} (17). The oven temperature was 50°C,

with detector and injector temperatures of 60°C and a filament current of 80 mA.

In experiments conducted in the presence of NO, appropriate volumes of 10-fold-diluted NO in argon were injected into incubations to reach the required final concentration of dissolved NO (its solubility in water was 1.8 mM under standard conditions).

Nitric oxide detection and measurement. Assays were carried out with argon in the gas phase, and at intervals of time, incubation mixtures (1.5 ml) were slowly withdrawn from the flasks with a syringe and injected into evacuated 5-ml "Venoject" tubes. NO in the evolved gas (500-μl aliquots) was determined with a gas chromatograph having a thermal conductivity detector and equipped with a Porapak R column (3 by 0.003 m). Nitrogen was the carrier with a flow rate of 45 ml min^{-1}; oven and detector temperatures were, respectively, 30 and 250°C, and the filament current was 140 mA (1).

RESULTS

Nitrite effects upon C_2H_2 reduction and H_2 evolution by purified nitrogenase. Time courses of nitrogenase activity showed a strong inhibitory effect of $NaNO_2$ upon C_2H_2 reduction, and the activity decreased rapidly with increasing NO_2^- concentrations (Fig. 1). Inhibition of 50% was reached with 0.1 mM $NaNO_2$ as previously reported (16).

In the same experiments, but in the absence of C_2H_2, the rate of H_2 evolved by nitrogenase incubations was linear with time, reaching 60 nmol min^{-1} mg^{-1}. Addition of $NaNO_2$ in the range previously used (Fig. 1) did not significantly modify H_2 evolution during the considered period of time (Fig. 2). A higher level of NO_2^- (1 mM) did not affect the reaction during the first 5 min but was responsible for a strong inhibition (40%) during the following 10 min.

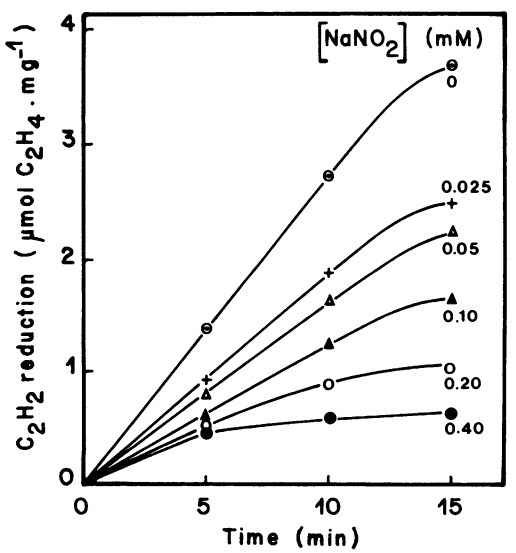

FIG. 1. Time courses of C_2H_2 reduction by purified nitrogenase in the presence of different concentrations of $NaNO_2$. Assays containing 2.2 mg of protein were shaken at 100 rpm.

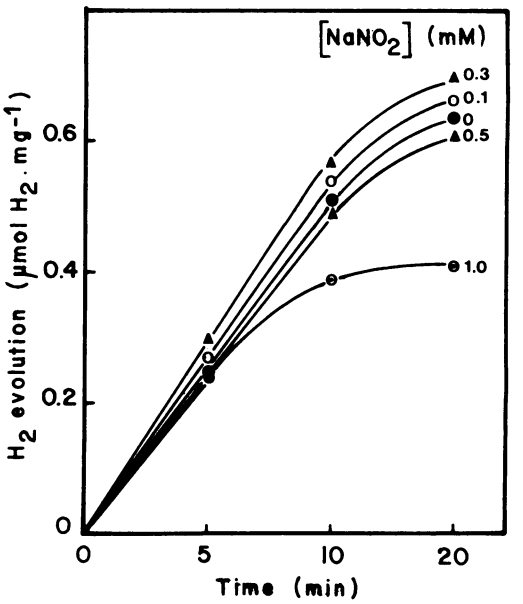

FIG. 2. Time courses of H_2 evolution by purified nitrogenase incubated with a range of $NaNO_2$ concentrations. Assays were performed as described in the text, with a protein content of 3.5 mg.

Nitric oxide inhibition of C_2H_2 reduction and H_2 evolution by purified nitrogenase. Addition of NO to nitrogenase incubations induced a drop in C_2H_4 formation. Experiments were performed in the presence of increasing concentrations of C_2H_2 and dissolved NO. The double-reciprocal plot representation (Fig. 3) showed that the apparent maximum velocity (V_{max}) decreased with increasing NO concentrations and that the apparent Michaelis constant (K_m) value of nitrogenase for C_2H_2 (0.05 mM) was unaffected by NO. This type of inhibition was referred to as noncompetitive, and the apparent inhibition constant (K_i) was 0.056 mM.

When C_2H_2 was omitted, H_2 evolution by nitrogenase preparations was followed with time and appeared strongly inhibited by NO. Figure 4 shows a typical inhibition curve when the rate of reaction was plotted for the first 10 min. At 0.01 mM NO concentration, 50% inhibition occurred. Thus, sensitivity of nitrogenase to NO appeared much more pronounced during H_2 evolution than during C_2H_2 reduction.

Nitric oxide generation in the presence of nitrite. Experiments were carried out by reference to the usual technique for generation of NO by the reduction of NO_2^- with a few crystals of dithionite (7, 13). NO was detected by gas

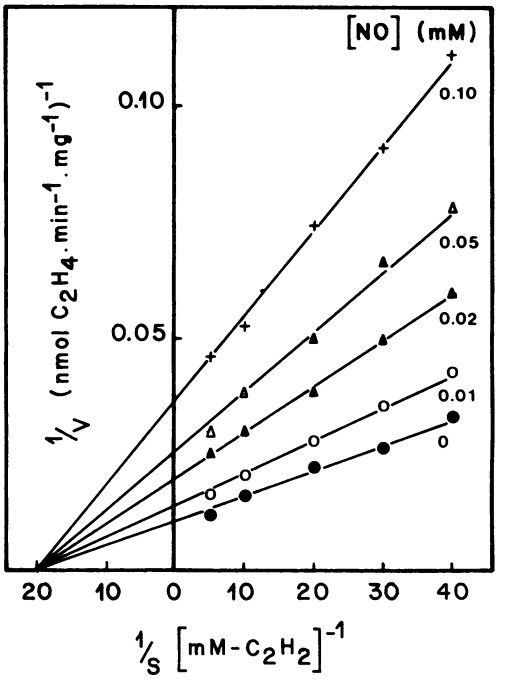

FIG. 3. Lineweaver-Burk plots for C_2H_2 at several constant concentrations of nitric oxide. Assays (2.8 mg of protein) were conducted for 10 min with shaking (100 rpm).

chromatography after degassing the incubation mixtures containing the usual concentration of dithionite and increasing levels of $NaNO_2$. In the range of 0.1 to 0.3 mM, no NO was detected after 30 min, but its presence could be observed when $NaNO_2$ concentrations were, respectively, 0.5 and 1 mM after a lag time of 10 and 5 min (Fig. 5). Under these conditions, 0.04 and 0.22 μM concentrations of NO were produced within the first 15 min. Higher levels of NO were generated by increasing $NaNO_2$ or dithionite concentrations. It can be noticed that our usual conditions for measuring nitrogenase activity, i.e., short periods of time for the experiments (5 to 15 min) and low levels of $NaNO_2$ (0.025 to 0.4 mM) and dithionite (10 mM), strongly limited NO formation in the assays.

DISCUSSION

Nitrite appeared as a strong inhibitor of C_2H_2 reduction by bacteroid nitrogenase, confirming our previous results (16), but H_2 evolution was not inhibited by nitrite under the same conditions. Similar results occurred with carbon monoxide, another common inhibitor of nitrogenase (12). Like CO, nitrite was unable to act as an alternative substrate for nitrogenase and also induced a reversible inhibition (16). In contrast, NO strongly inhibited both C_2H_2 reduction and H_2 evolution by bacteroid nitrogenase according to the recent results obtained with *Clostridium* enzyme (6). The K_i value determined here for NO (0.056 mM) was very low compared with that previously reported for NO_2^- (5.2 mM), pointing out a very high sensitivity of nitrogenase to NO. Another difference concerning the type of inhibition was observed, since NO_2^- competitively inhibited C_2H_2 reduction (16) whereas inhibition by NO was referred to as noncompetitive. Thus, these results confirmed that NO_2^- and NO were able to react with nitrogenase in a characteristic and independent manner. However, the possibility for NO_2^- to inhibit nitrogenase through NO, its reduction product, was recently proposed (6). Dithionite universally added as a reductant to nitrogenase assays was indeed able to contribute to this reduction. Under our experimental conditions, we reported a low level of NO produced from NO_2^- (Fig. 5), in a range where nitrogenase was insensitive (Fig. 4), ruling out a direct intervention of NO in the inhibitory effect of NO_2^- reported in this paper.

An explanation for the discrepancy between our results and those reported by Meyer (6) can be found in the procedure adopted for the nitrogenase assays by this author and favoring NO generation: the reactions were initiated by MgATP after 5 min of preincubation of the

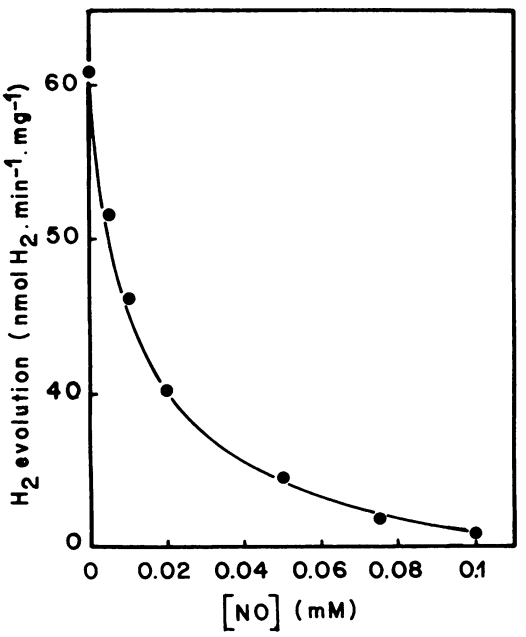

FIG. 4. Inhibitory effect of NO upon H_2 evolution by purified nitrogenase. Incubations were carried out for 10 min with a protein content of 3.5 mg under conditions described in the text.

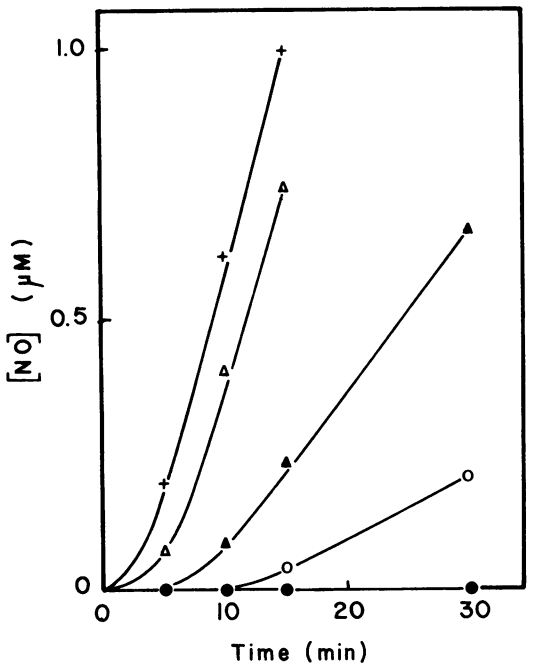

FIG. 5. Nitric oxide production by reduction of nitrite by dithionite. Incubation mixtures (1.5 ml) contained 10 mM dithionite and 0.1 to 0.3 (●), 0.5 (○), 1 (▲), or 2 (△) mM $NaNO_2$ or 15 mM dithionite and 1 mM $NaNO_2$ (+). The flasks were placed at 30°C with shaking (100 rpm).

mixture containing nitrite and a high level of dithionite (15 mM). Thus, we observed that 1 mM NO_2^-, unable to modify H_2 evolution during the first 5 min (Fig. 2), induced an increasing inhibition for the following 10 min. The conjugated effect of high NO_2^- concentration and a longer period of time enhanced the generation of NO, a powerful inhibitor of H_2 evolution. The lag time observed in this phenomenon can also be compared with those occurring during the NO formation under the same conditions (Fig. 5). Relatively high levels of NO were previously reported by Meyer (6), resulting from NO_2^- reduction by dithionite, in long-term experiments. NO_2^- disappearance was used to determine NO concentrations, but the results could be questioned since incomplete oxidation of dithionite interfered with the colorimetric assays (5, 15) inducing a 50% overestimation of NO (J. C. Trinchant, unpublished data).

Thus, nitrite detected in vivo after feeding legumes with NO_3^- and resulting from a nitrate reductase activity located in bacteroids (8) or in nodule cytosol (2) could directly act in inhibiting nitrogenase activity. The validity of this hypothesis seems strengthened since the possibility of a carbohydrate deprivation of nodules, following a nitrate treatment, appeared less consistent after the recent results reported by Streeter (14).

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