Regulation of Nitrogen Fixation in *Rhodospirillum rubrum* Grown Under Dark, Fermentative Conditions

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*Rhodospirillum rubrum* was shown to grow fermentatively on fructose with N₂ as a nitrogen source. The nitrogenase activity of these cells was regulated by the NH₄⁺ switch-off/switch-on mechanism in a manner identical to that for photosynthetically grown cells. In vitro, the inactive nitrogenase Fe protein from fermenting cells was reactivated by an endogenous membrane-bound, Mn²⁺-dependent activating enzyme that was interchangeable with the activating enzyme isolated from photosynthetic membranes.

The purple photosynthetic bacteria have a system for the regulation of nitrogenase activity that is not commonly found in heterotrophic bacteria. In this system, NH₄⁺ inhibits nitrogenase activity in both *Rhodospirillum rubrum* (2, 11, 15) (and all other members of the family *Rhodospirillaceae* tested) and *Chromatium vinosum* cells (5). The inhibition, which is readily reversed when the NH₄⁺ has been metabolized by the cells, is commonly called the NH₄⁺ "switch-off/switch-on" effect (19). A covalent modification of the nitrogenase Fe protein (7) has been shown to account for NH₄⁺ switch-off in *R. rubrum* (1, 12), *Rhodopseudomonas capsulata* (10), and *C. vinosum* (5) cells. In *R. rubrum* cells, the appearance of a modified subunit of Fe protein on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels was shown to correlate with the loss of Fe protein activity (3, 13).

A recent report (6) suggests that darkness has the same effect as NH₄⁺ in initiating the modification (and inhibition) of the Fe protein in photosynthetically grown *R. rubrum* cells. Since Madigan et al. (9) had already shown that a related species, *Rhodopseudomonas capsulata*, was capable of growing anaerobically in the dark on N₂, darkness obviously had no inhibitory effect on the nitrogenase of this species, whose growth was supported by an oxidant-dependent sugar fermentation. Furthermore, NH₄⁺ (2-h exposure) inhibited C₂H₂ reduction by this organism. The objective of this study was twofold. First, we wanted to determine whether *R. rubrum* S1 cells (from the Indiana University collection) were capable of growing on N₂ under fermentation conditions in the absence of an accessory oxidant. Secondly, in light of the observations that *R. rubrum* nitrogenase Fe protein was switched-off in cells placed in the dark, we wanted to know whether this enzyme (if expressed in the cells fermentatively grown in the dark) was also regulated by the NH₄⁺ switch-off/switch-on regulatory system.

The ability of *R. rubrum* cells to grow fermentatively on fructose with NH₄⁺ has already been established by Schultz and Weaver (16), and their ability to grow fermentatively with N₂ as a nitrogen source is shown in Fig. 1. Although the growth rate was slow compared with growth on NH₄⁺ (doubling time, 70 versus 27 h), it clearly demonstrates that

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N₂ fixation occurs in photosynthetic bacteria in which energy and reductant for growth are both supplied by a strict (nonoxidant-dependent) fermentative process. Nitrogenase activity in these fermenting cultures was confirmed by the acetylene reduction assay (Fig. 2), in which, after a short lag period, maximum rates of ethylene production (60 nmol h⁻¹ mg [dry wt]⁻¹) were observed.

The addition of 2 mM NH₄⁺ to one of the cultures (Fig. 2) immediately decreased the rate of nitrogenase activity (in relation to a control to which water was added) until it reached zero about 1.5 h after the addition of the inhibitor.

**TABLE 1. Requirement for activation of nitrogenase from fermentatively grown *R. rubrum* cells**

<table>
<thead>
<tr>
<th>Reaction mixture</th>
<th>Sp act (nmol of C₂H₄ formed min⁻¹ mg of protein⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete*</td>
<td>25.6</td>
</tr>
<tr>
<td>Minus AE</td>
<td>0.1</td>
</tr>
<tr>
<td>Minus Mn²⁺</td>
<td>0.1</td>
</tr>
<tr>
<td>Minus AE and Mn²⁺</td>
<td>0</td>
</tr>
</tbody>
</table>

* Nitrogenase was extracted from cells grown in the dark on fructose with 5 mM glutamate as a nitrogen source. Extracts were prepared anaerobically by sonication as previously described (18).

**FIG. 2.** Effect of NH₄⁺ on acetylene reduction by *R. rubrum* cells growing fermentatively on fructose and N₂. At time zero, 10% acetylene was added to mid-log cultures, and at the time indicated by the arrow 10 µl of distilled water (a control) or 10 µl of 2 mM NH₄Cl (final concentration) was added. The curves represent the average values for C₂H₄ reduction of duplicate cultures in the dark. Ethylene was detected by flame ionization gas chromatography by using a Porapak N column maintained at 62°C (detector, 200°C).

The inhibition by NH₄⁺ suggests that nitrogenase activity in cells fermenting in the dark is regulated by a switch-off/switch-on mechanism similar to that found in cells anaerobically grown in the light, for which NH₄⁺ catalyzes the covalent modification (i.e., inactivation) of the Fe protein. Further evidence of this covalent modification system is seen in Table 1, which shows that a partially purified nitrogenase preparation has a complete dependence on both the activating enzyme (AE), the enzyme which is known to remove the modifying molecule from the Fe protein (8), and Mn²⁺, a cofactor for this enzyme. In this experiment AE was prepared from photosynthetically grown cells, but AE from fermenting cells worked equally well (data not shown). We found that in crude nitrogenase extracts it was difficult to demonstrate more than a twofold stimulation of activity by AE, since these extracts were usually contaminated with low levels of this enzyme.

The isolation of a functional nitrogenase AE from extracts of cells grown fermentatively (Fig. 3) indicates that these cells are fully capable of regulating their nitrogenase by the covalent modification mechanism. AE was solubilized as described previously (7) from the cell membranes (the pellet obtained after centrifugation of the crude extract at 250,000 x g for 90 min) by washing them in 0.5 M NaCl. The AE in the washing solution was concentrated as a 10 to 30% polyethylene glycol precipitate. Both the membrane preparation and the solubilized fraction showed good AE activity when tested on partially purified Fe protein from photosynthetic cells, which is known to be regulatable. Higher concentrations of membranes appeared to contain a substance that inhibited complete activation of Fe protein (Fig.
3, solid circles), but removal of AE from the membranes removed the inhibitory effect and increased the degree of Fe protein activation (open circles). This AE preparation was equally effective in activating Fe protein from cells grown in the light.

In summary, we have demonstrated that R. rubrum cells grow in the dark fermentatively on fructose and are capable of supporting themselves by nitrogen fixation under these conditions. Along with the observations of Voelskow and Schön (17), who demonstrated H₂ evolution from nitrogenase in pyruvate-fermenting R. rubrum cells, and Madigan et al. (9), who showed that Rhodopsseudomonas capsulata cells can grow anaerobically in the dark on N₂ supported by a fructose-dimethyl sulfoxide coupled metabolism, the data reported here indicate that nitrogenase in fermenting cells is functional in the dark and is therefore apparently unlike that of cells grown in the light, where Fe protein was inactivated by the cofactual modification mechanism when placed in the dark (6). It is possible that the Fe protein inactivating enzyme (which has yet to be demonstrated in vitro) is itself regulated by either the redox environment or high-energy molecules such as ATP. The observations cited above suggest that a fermentative metabolism can provide the proper environment for maintaining the Fe protein in its active state, whereas photosynthetically grown cells placed in the dark cannot maintain this environment and Fe protein is inactivated. Alternatively, the Fe protein in fermenting R. rubrum cells in the dark may be predominantly inactive (as was reported for cells grown in the light when placed in the dark), but the 5 to 10% which remains active is sufficient to provide enough fixed nitrogen for these cells to grow in the dark at the rates seen in Fig. 1. Because of the spontaneous inactivation of Fe protein that occurs on disrupting R. rubrum cells, it is difficult to determine the ratio of active to inactive Fe protein that exists in vivo.

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LITERATURE CITED


