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STRUCTURAL INTERACTION BETWEEN NITROGENASE AND CELL MEMBRANE IN FREE-LIVING, NITROGEN-FIXING BACTERIA

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Department of Microbiology

by

Karen Smith Howard
B.S., University of Alabama, 1970
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENT</td>
<td>ii</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>iii</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>iv</td>
</tr>
<tr>
<td>LIST OF ABBREVIATIONS</td>
<td>v</td>
</tr>
<tr>
<td>ABSTRACT</td>
<td>vi</td>
</tr>
<tr>
<td>PART I. Nitrogen Fixation and Ammonia Switch-off in the Photosynthetic Bacterium <em>Rhodopseudomonas viridis</em></td>
<td>1</td>
</tr>
<tr>
<td>Introduction</td>
<td>2</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>5</td>
</tr>
<tr>
<td>Results</td>
<td>8</td>
</tr>
<tr>
<td>Discussion</td>
<td>17</td>
</tr>
<tr>
<td>Literature Cited</td>
<td>22</td>
</tr>
<tr>
<td>PART II. <em>In Vivo</em> Interaction Between Nitrogenase Molybdenum-Iron Protein and Membrane in <em>Azotobacter vinelandii</em> and <em>Rhodospirillum rubrum</em> as Observed by Electron Spin Resonance Spectroscopy</td>
<td>27</td>
</tr>
<tr>
<td>Introduction</td>
<td>28</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>32</td>
</tr>
<tr>
<td>Results</td>
<td>38</td>
</tr>
<tr>
<td>Discussion</td>
<td>54</td>
</tr>
<tr>
<td>Literature Cited</td>
<td>62</td>
</tr>
<tr>
<td>VITA</td>
<td>68</td>
</tr>
</tbody>
</table>

**iii**
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PART I.</strong></td>
<td></td>
</tr>
<tr>
<td>1. <em>R. viridis</em> growth and acetylene reduction rates in batch culture</td>
<td>9</td>
</tr>
<tr>
<td>2. Negative stain of <em>R. viridis</em> ATCC 19567 grown under nitrogen-fixing conditions</td>
<td>11</td>
</tr>
<tr>
<td>3. First-derivative esr spectra of <em>R. viridis</em> and <em>R. palustris</em> whole cells grown under nitrogen-fixing conditions</td>
<td>13</td>
</tr>
<tr>
<td>4. Ammonia switch-off of acetylene reduction in <em>R. viridis</em> and <em>R. palustris</em></td>
<td>15</td>
</tr>
<tr>
<td>5. A comparison of collected nitrogenase g factors with the theoretical values of a spin 3/2 system</td>
<td>19</td>
</tr>
<tr>
<td><strong>PART II.</strong></td>
<td></td>
</tr>
<tr>
<td>1. Preparation of membrane multilayers</td>
<td>35</td>
</tr>
<tr>
<td>2. Variations in whole cell <em>A. vinelandii</em> esr signals</td>
<td>39</td>
</tr>
<tr>
<td>3. Orientation dependent ((\text{BChl} \ a)_2^T) spectra in <em>R. rubrum</em> MM's</td>
<td>43</td>
</tr>
<tr>
<td>4. Orientation dependent ((\text{BChl} \ a)_2^T) spectra in <em>R. rubrum</em> WCM's</td>
<td>45</td>
</tr>
<tr>
<td>5. Orientation dependent 5-doxyl stearate spectra in <em>A. vinelandii</em> MM's</td>
<td>46</td>
</tr>
<tr>
<td>6. Orientation dependent 5-doxyl stearate spectra in <em>A. vinelandii</em> WCM's</td>
<td>47</td>
</tr>
<tr>
<td>7. Angular dependence of nitrogenase MoFe protein esr signals in <em>R. rubrum</em> WCM's</td>
<td>49</td>
</tr>
<tr>
<td>8. Angular dependence of nitrogenase MoFe protein g3.6 signal in <em>A. vinelandii</em> WCM's</td>
<td>50</td>
</tr>
<tr>
<td>9. Variations in the angular dependent properties of the nitrogenase MoFe protein g4.3 signal in <em>A. vinelandii</em> WCM's</td>
<td>53</td>
</tr>
</tbody>
</table>
LIST OF ABBREVIATIONS

ABBREVIATION

Bchl a .................. Bacteriochlorophyll a
(Bchl a)₂^... Bacteriochlorophyll a dimer triplet state
Bchl b .................. Bacteriochlorophyll b
Esr ..................... Electron spin resonance
FeMoco .................. Iron-molybdenum cofactor of the nitrogenase MoFe protein
Fe protein .............. Nitrogenase iron protein (Component II)
MoFe protein .......... Nitrogenase molybdenum-iron protein (Component I)
MM ........................ Membrane multilayer
WCM ........................ Whole cell multilayer
Rhodopseudomonas viridis ATCC 19567 grows by means of nitrogen fixation in nitrogen-limited or nitrogen-free medium when sparged with 5% CO₂ and 95% N₂ in the light at 30°C. Growth characteristics of batch cultures were very consistent. Acetylene reduction assays for nitrogenase activity revealed an initially high level of activity during early-logarithmic growth phase, a lower plateau during mid- to late-logarithmic phase, and a dramatic reduction of activity at the beginning of stationary phase. When viewed by electron microscopy, nitrogen-fixing R. viridis cells appeared to be morphologically and ultrastructurally similar to cells grown on nitrogen-rich media. Whole cells prepared under reducing conditions in the dark for electron spin resonance spectroscopy yielded g₄.26 and g₃.66 signals characteristic of the molybdenum-iron protein of nitrogenase. During growth on N₂ in the absence of fixed-nitrogen sources, the nitrogenase activity of R. viridis measured by acetylene reduction stopped rapidly in response to the addition of NH₄Cl in a manner similar to that observed in other Rhodospirillaceae. However, unlike the nitrogenase of Rhodopseudomonas palustris or Rhodospirillum rubrum which recover from this treatment within 40 minutes, nitrogenase activity in R. viridis was not detectable for nearly 4 h.
ABSTRACT - PART II

Electron spin resonance (esr) spectroscopy of oriented whole cell multilayers (WCM's) of Azotobacter vinelandii and Rhodospirillum rubrum was used to detect structural associations between nitrogenase molybdenum-iron protein (MoFe protein) and cytoplasmic or intracytoplasmic membrane. Conditions were defined for observing MoFe protein esr signals in whole cell preparations of each organism. The orientation of membranes in A. vinelandii WCM's was demonstrated using doxyl stearate spin label and orientation of intracytoplasmic membrane was demonstrated in R. rubrum WCM's by the orientation dependence of the bacteriochlorophyll a dimer triplet signal, \((\text{BChl a})_2^T\), from the intracytoplasmic membrane-bound primary donor unit. The low field MoFe protein signals, \(g_{4.3}\) and \(g_{3.6}\), showed orientation dependent characteristics in WCM's of both organisms, although the properties of each were not identical. As the normal to the membrane plane was rotated from perpendicular to parallel with the esr magnetic field, the amplitude of the \(g_{3.6}\) signal decreased from maximum to 37% of maximum in A. vinelandii, and from maximum to 88% of maximum in R. rubrum. The angular dependence of the \(g_{4.3}\) peak during rotation varied in A. vinelandii, but decreased from maximum to 63% of maximum in R. rubrum. These properties suggest that the MoFe protein of nitrogenase was oriented in response to the physical orientation of cellular membranes and that a structural association exists between this nitrogenase component and membrane in these organisms.
PART I

Nitrogen Fixation and Ammonia Switch-Off
in the Photosynthetic Bacterium *Rhodopseudomonas viridis*
INTRODUCTION

All members of the family Rhodospirillaceae that have been tested have been shown to fix nitrogen (31). In these organisms, nitrogen fixation usually occurs photosynthetically under anaerobic conditions although low levels of nitrogen fixation have been demonstrated in the dark during microaerophilic or fermentative growth (21,30). Nitrogen reduction occurs as a result of the concerted interaction of an iron protein (Fe protein) and a molybdenum-iron protein (MoFe protein) whose properties are similar to the nitrogenase components of non-photosynthetic, nitrogen-fixing bacteria (18). These proteins have been isolated in purified form (4) from Rhodospirillum rubrum, one of the more extensively studied members of this family. Unlike the Fe protein from non-photosynthetic bacteria, however, the Fe protein of R. rubrum can be isolated in either an active or inactive form (17,24,4) which is due to the absence or presence, respectively, of a covalently bound modifying group (18,19,20,13). Inactive Fe protein can be activated in vitro by an intracytoplasmic membrane-bound enzyme in conjunction with divalent metal ion and ATP (17).

In vivo nitrogenase activity in several Rhodospirillaceae is subject to short-term regulation by certain fixed-nitrogen-containing compounds (1), a phenomenon known as ammonia switch-off (37). Although the mechanism of ammonia switch-off is unknown, a direct relationship has been suggested between this event in vivo and the observed modification of the Fe protein (4,13).

There has been little characterization of nitrogen fixation in Rhodopseudomonas viridis, also a member of Rhodospirillaceae. In 1971, Pratt, Bergad, and Ham reported some nitrogen-fixing properties of a
Rhodopseudomonas isolate identified as *R. viridis* and, in a review article in 1981, Postgate listed it as a nitrogen-fixer. We have further characterized the nitrogen fixation and ammonia switch-off properties of this species because of several features which make *R. viridis* unusual among the purple, non-sulfur bacteria. First, it is one of two members which contain bacteriochlorophyll b (BChl b) in its photosynthetic reaction centers instead of bacteriochlorophyll a (BChl a). Since BChl b in reaction centers absorbs light of longer wavelengths than BChl a, the amount of energy in the first excited singlet state of *R. viridis* is less than that in other Rhodospirillaceae (28). Thus, the energy coupling between photosynthesis and nitrogen fixation in this organism may differ from the BChl a-containing species. Second, *R. viridis* bears a similarity in size, shape, and thylakoid structure to *Rhodopseudomonas palustris*, another member of the Rhodospirillaceae (33). However, electron microscopic examination of the intracytoplasmic membrane supramolecular organization of *R. viridis* reveals a nearly crystalline periodicity unlike the photosynthetic membranes of purple, non-sulfur bacteria (12,25).

In summary, *R. viridis* ATCC 19567 was capable of growth on N$_2$ as its only nitrogen source. The rate of nitrogenase activity was measured throughout growth in batch culture by acetylene reduction. Electron spin resonance (esr) spectroscopy of whole cells revealed spectra indicative of the presence of a conventional MoFe nitrogenase protein in the g3.6 and g4.3 regions (6). Electron microscopic examinations of nitrogen-fixing and non-nitrogen-fixing organisms showed similarities in their morphology and the extent of their intracytoplasmic membrane development. Finally, we demonstrated ammonia switch-off events in
nitrogen-fixing cultures of *R. viridis*, but could not demonstrate switch-on in a period of time less than approximately 4 h.
MATERIALS AND METHODS

Organisms and growth conditions. Unless otherwise specified, *Rhodopseudomonas viridis* (ATCC 19567) and *Rhodopseudomonas palustris* (ATCC 17001) used in these experiments were grown anaerobically, at 30°C, under incandescent light of ca. 6 mW/cm² intensity. Stock cultures of *R. viridis* (9) and *R. palustris* (11) were grown as previously described in media containing yeast extract as a nitrogen source and were transferred every 14-21 days. In order to derepress nitrogenase, batch cultures of both organisms were grown in 200 ml volumes of the nitrogen-limited medium of Pfenning (23), which contains sodium succinate and 0.1% yeast extract, and were continually sparged with 95% N₂ and 5% CO₂.

Cell growth analysis. Absorption spectra were recorded of early-, mid-, and late-logarithmic phase cultures between 500-900 nm using a Cary 219 (Varian) spectrophotometer. Since no absorption peaks were observed at 660 nm for either bacterium, turbidity of batch cultures was monitored at this wavelength using a Klett-Summerson colorimeter. The colorimeter was calibrated using dilutions of late-logarithmic phase cultures to correct for loss of sensitivity at high concentrations of cells (15). The reported growth curves were corrected for this deviation and the data converted to absorbency units (A).

Protein determination. Following acetone-methanol extraction of pigments (5) total cell protein was determined by the Lowry method (16) using bovine serum albumin as the standard. Using this method, the relative protein concentration (µg of protein per ml of whole cells per A) was found to remain essentially constant throughout the period of growth. Relative protein concentrations of 890 for *R. palustris* and 442
for *R. viridis* were determined by a least squares fit of the data
designating µg protein *· ml whole cells⁻¹ as a function of A obtained at
various times during growth of the organisms in batch cultures.

**Acetylene reduction assay.** Acetylene reductions were performed by
a modification of the Burris method (3). A 15 min preincubation of 10
ml cell samples in 50 ml septum bottles was conducted under an Ar
atmosphere in the light (6 mW/cm²) at 30°C to allow temperature and gas
equilibration. The concentration of acetylene used was 10 kPa. The
length of incubation varied according to experimental conditions.

**Electron microscopy.** Negative stains of cells of *R. viridis* in
mid-logarithmic growth stage were prepared with 0.25% ammonium molybdate
on carbon stabilized, Formvar coated, 200 mesh copper grids. The cells
were photographed in a JEOL-100CX electron microscope at 80 kV.

**Electron spin resonance spectroscopy.** Esr samples of mid-
logarithmic growth stage cells were prepared similarly to previously
published methods (10). Throughout the procedure all solutions were Ar-
flushed for 3-5 min prior to use. Cultures were harvested at 8000 x g
for 10 min in Ar-flushed centrifuge bottles, resuspended in 0.025 M tris
(hydroxymethyl) aminomethane-HCl buffer (pH 7.4), and recentrifuged at
8000 x g for 10 min. From this point until frozen, samples were kept in
darkness or dim light. After centrifugation, the cells were resuspended
under Ar in an equal volume of 0.10 M glycylglycine buffer (initial pH
10.1 with KOH) containing 0.10 M KCl, 1.0 x 10⁻⁴ M methyl-viologen, plus
freshly added 0.040 M Na₂S₂O₄ (final pH 8.0). This suspension was
further diluted by one half with an equal volume of glycerol and
thoroughly mixed with a stream of Ar delivered from a Pasteur pipette.
The cells were then transferred to Ar-flushed quartz esr tubes, capped,
and frozen within one minute with liquid nitrogen cooled N₂ and stored in liquid nitrogen. Esr spectra were observed at X-band wavelengths with either a Varian E12 spectrometer using an Oxford liquid He cryostat (Princeton University) or a Varian E-109 ES spectrometer using a Helitrans liquid He cryostat (Air Products, Inc., Model LTD-3-110) at temperatures between 4-9K.

**Chemicals.** All growth media were prepared with reagent grade salts, Bacto yeast extract (Difco Laboratories), and sodium succinate (Nutritional Biochemicals Corp.) or ammonium succinate (ICN Pharmaceutical, Inc.). Buffer components included reagent grade TRIZMA, pH 7.4 (tris (hydroxymethyl) aminomethane-HCl) or glycylglycine (both Sigma), reagent grade salts, methyl-viologen (Mann Research Laboratories, Inc.), and sodium dithionite (Sigma).
RESULTS

Nitrogen fixation by whole cells. *Rhodopseudomonas viridis* ATCC 19567 was capable of continued photosynthetic growth at 30°C after transfer from nitrogen-rich stock medium to nitrogen-limited medium containing 0.1% yeast extract when supplied with 95% N₂ and 5% CO₂. Growth of a 200 ml batch culture (Fig. 1) proceeded for 82 to 85 h before entering the stationary phase at a turbidity of ca. 0.5 A. The stationary phase turbidity and the 65 to 70 h logarithmic growth phase were consistent characteristics of nitrogen-fixing batch cultures of *R. viridis*, which had a generation time of 4.5 h as compared to 1.75 h for *R. palustris* grown under identical conditions. *R. viridis* transferred during mid-logarithmic growth from original Pfennig's medium to Pfennig's medium free of yeast extract showed no alteration in growth rate as compared to cells held in the original medium (data not shown). These data suggest that the cells in original Pfennig's medium had utilized the nitrogen available in yeast extract by mid-logarithmic growth phase and did not require it for continued growth while fixing nitrogen in nitrogen-free medium.

Figure 1 shows the rate of nitrogen-fixing activity (nM C₂H₄ produced per min per mg of whole cell protein) during *R. viridis* growth in batch culture. Ethylene was not detectable in samples taken during the lag or initial logarithmic growth phase of cultures inoculated with either nitrogen-rich or nitrogen-limited stocks. This may have been due either to lack of sufficient cell numbers to produce detectable amounts of ethylene during the 15 min incubation time or to repression of nitrogenase by the initial fixed-nitrogen sources present in the medium. The rate of ethylene production was greatest during early-
Figure 1. *R. viridis* growth and acetylene reduction rates in batch culture. A 1% inoculum of *R. viridis* stock culture grown in nitrogen-rich medium was transferred to 200 ml of nitrogen-limited medium, grown, and monitored turbidimetrically as described in Materials and Methods. Turbidity was corrected as described above. Acetylene reductions were performed with duplicate 10 ml samples taken from duplicate 200 ml batch cultures such that each data point is the average of four samples. Standard deviation among the samples is denoted by bars. Following a 15 min incubation for acetylene reduction, each sample was frozen and used to determine mg whole cell protein per ml of culture per A unit. •, turbidity; ○, rate of ethylene production.
logarithmic phase, decreased mid- to late-logarithmic phase, and was marked by an immediate reduction of activity at the beginning of stationary phase.

Acetylene reduction appeared to be reversibly inhibited by exposure to small amounts of O$_2$ as reported in other photosynthetic bacteria (14). Occasionally, one of a duplicate or triplicate set of samples would be inactivated following transfer from the batch culture to the reaction vessel, presumably due to exposure to air. However, the inactive sample usually regained its acetylene reducing activity after an hour or more of incubation, suggesting that *R. viridis* nitrogenase was protected upon exposure to very low concentrations of O$_2$ even though the organism is reportedly a strict anaerobe (7).

**Electron microscopy of nitrogen-fixing cells.** We were interested in comparing the morphology and ultrastructure of *R. viridis* growth in both nitrogen-enriched and nitrogen-limited media. Negative stains and thin sections of exponentially growing cells from 0.5% yeast extract and ammonium succinate enriched medium and nitrogen-limited medium were indistinguishable. Figure 2 shows a negative stain of nitrogen-fixing *R. viridis*. Typically, cells observed from mid-logarithmic phase cultures were in the process of budding. In negative stains, round, dense bodies appeared in all cells generally located at one or both poles. Longer cells often contained three bodies nearly equally spaced whereas shorter cells sometimes contained only one. The size of the dense bodies varied and could be reduced to some extent by beam damage in the electron microscope.

Thin sections of *R. viridis* ATCC 19567 revealed similar amounts of intracytoplasmic membrane development regardless of nitrogen source.
Figure 2. Negative stain of *R. viridis* ATCC 19567 grown under nitrogen-fixing conditions. The cells were removed from an exponentially growing culture in nitrogen-limited medium and observed by negative staining with 0.25% ammonium molybdate. Dense bodies were typically seen towards the poles of vegetative cells, but not in regions where buds appeared to be forming. Bar = 17 nm.
However, the extent of thylakoid development in this strain was significantly less than in another strain of R. viridis (gift of H. Frank) grown under identical conditions (G.C. Dismukes, personal communication) and observed by electron microscopy. Although both strains grew equally well under nitrogen-fixing conditions, they differed in two other respects. Our ATCC 19567 strain retained its yellow-green color regardless of the age of the culture and did not adhere to glassware, whereas the second R. viridis strain gradually developed a brownish color in aging cultures, as previously reported (7), and did adhere to the sides of growth vessels even in cultures constantly mixed by sparging gas.

**Electron spin resonance spectroscopy of whole cells.** Nitrogen-fixing R. viridis and R. palustris were prepared for esr spectroscopy in the presence of sodium dithionite and methyl-viologen in the dark. These conditions were used to enhance the $g_{4.3}$ and $g_{3.6}$ peaks associated with the active MoFe protein of nitrogenase by reducing background signals in this region of the whole cell esr spectra. Under these reducing conditions, R. viridis contained peaks at $g_{4.26}$ and $g_{3.66}$ comparable to signals observed from the nitrogenase MoFe protein in whole cells of other nitrogen-fixing bacteria, e.g. R. palustris (Fig.3 and 38) and Azotobacter vinelandii (6). In whole cells of all three organisms, the $g_{2.01}$ peak observed in purified MoFe protein is obscured by other strong signals in the $g_{2}$ region.

**Ammonia switch-off in R. viridis.** Numerous members of the Rhodospirillaceae family demonstrate a reversible loss of in vivo nitrogenase activity when exposed to NH$_4^+$ under specific nutritional and environmental conditions (4,35,31,36). This phenomenon has been
Figure 3. First-derivative esr spectra of *R. viridis* and *R. palustris* whole cells grown under nitrogen-fixing conditions. Organisms were dark adapted for approximately 15 min prior to reduction by Na$_2$S$_2$O$_4$. Samples were held in the dark until they were frozen to 77K for storage. The spectra were obtained under the following instrumental conditions: *R. viridis*; power 5.0 mW, frequency 9.0 GHz, modulation amplitude 12.5 G, time constant 1 sec, temperature 9K. *R. palustris*; power 5.0 mW, frequency 9.233 GHz, modulation amplitude 20 G, time constant 3 sec, temperature 4K. T, Tesla; H, magnetic field strength.
characterized most extensively in *R. rubrum*, although *R. palustris* shows similar characteristics in its response to \( \text{NH}_4^+ \) when growing by means of nitrogen fixation in the light on sparged \( \text{N}_2 \) \((38,35,1)\). Under these conditions, both organisms reversibly stop nitrogenase production of ethylene or hydrogen \((31)\) within minutes following addition of \( \text{NH}_4\text{Cl} \). Furthermore, the length of the inactive period in *R. palustris* has been shown to depend on the amount of \( \text{NH}_4\text{Cl} \) added \((38)\). Once the ammonia concentration in the medium drops below 0.10 mM, nitrogenase activity rapidly resumes. Since *R. palustris* and *R. viridis* are closely related species \((33)\), we chose to compare ammonia switch-off of nitrogenase activity between these two organisms.

Figure 4 shows the effect of 0.20 mM \( \text{NH}_4\text{Cl} \) on ethylene production in exponentially growing, nitrogen-fixing cultures of *R. palustris* and *R. viridis*. Duplicate 10 ml samples of each culture were transferred anaerobically to 50 ml septum bottles under an Ar atmosphere. Following a 15 min preincubation, acetylene (10 kPa) was added at time zero. An Ar-flushed \( \text{NH}_4\text{Cl} \) solution (0.4 ml, 5.0 mM) was added at the times indicated by arrows (Fig. 4) to produce a final concentration of 0.20 mM. *R. palustris* nitrogenase responded in the manner previously reported by Zumft and Castillo \((38)\) by rapidly halting production of ethylene and then resuming its activity after approximately 35 min. Under the same conditions, *R. viridis* nitrogenase was rapidly inactivated within 5 min, but did not resume for nearly 4 h.

Approximately 4 h after the addition of ammonia, the amount of ethylene in the sample had increased from 120 nM mg protein\(^{-1}\) to 220 nM mg protein\(^{-1}\). During the same time span, ethylene in the control sample increased at a nearly linear rate to 780 nM mg protein\(^{-1}\). Finally,
Figure 4. Ammonia switch-off of acetylene reduction in *R. viridis* and *R. palustris*. These data from a single experiment are representative of four *R. viridis* and two *R. palustris* experiments, each run in duplicate. The absorbancies of the batch cultures at the time this experiment was begun were 0.235 for *R. viridis* and 0.185 for *R. palustris*. Ar-flushed NH₄Cl was added by syringe to a final concentration of 0.20 mM at the times indicated by the arrows. •, *R. viridis* treated with NH₄Cl; ■, *R. palustris* treated with NH₄Cl; ○, *R. viridis* control (line was determined by a weighted least squares fit of the data points).
increasing concentrations of NH$_4$Cl up to 0.10 M did not lengthen the period of inactivation.
DISCUSSION

*Rhodopseudomonas viridis* grew vigorously due to the production of a nitrogen-fixing system when supplied with initially low concentrations of yeast extract and continuous N$_2$. Once acetylene reduction capability was present in a batch culture, the cells could be anaerobically transferred to medium free of yeast extract and growth would continue in the presence of N$_2$ as the only nitrogen source. When nitrogenase activity was measured by means of acetylene reduction during growth of a batch culture, the highest rate of activity was observed in early-log phase. This observation is consistent with other data which suggest that nitrogenase derepression is greatest at this time because of the lack of high concentrations of nitrogenase-produced ammonia in the cells. Once enough nitrogenase has been made to surpass the growth requirements of the cells, any pool of excess ammonia or intermediates in the nitrogen utilization pathway may cause partial repression of the nif operon and, hence, reduce nitrogenase activity (6).

It is also possible, however, that the unidentified mechanism responsible for ammonia switch-off in the Rhodospirillaceae represents a finer control than repression for nitrogenase activity, which may be partially regulated by fluctuating endogenous ammonia concentrations in addition to exogenously supplied ammonia.

Upon entering stationary growth phase, *R. viridis* nitrogenase activity was rapidly reduced to a very low level, although not completely inactivated. The loss of activity and the initiation of stationary phase occurred within 5 h. This trait is unlike *R. palustris* in which both nitrogenase activity and ammonia switch-off can be demonstrated in stationary phase (1).
Electron microscopic examination of *R. viridis* revealed no obvious differences between cells grown with fixed-nitrogen versus N₂. Cells of similar size and shape, as well as actively budding organisms, were evident in both types of media. No long chains or involution forms were apparent. Growth under identical light conditions caused the development of similar quantities of intracytoplasmic membrane regardless of the nitrogen source in strain ATCC 19567. However, we found that growth of a second strain under identical conditions resulted in a much greater proliferation of the photosynthetic membranes.

Esr spectra of *R. viridis* whole cells prepared in dim light with sodium dithionite were comparable to the esr spectra of whole, nitrogen-fixing *R. palustris*, *Azotobacter vinelandii* and *R. rubrum* observed in our laboratory and others (Fig. 5). Under these conditions, *R. viridis* and *R. palustris* spectra contained peaks at \( g_{4.26} \) and 3.66 and \( g_{4.24} \) and 3.65, respectively, indicative of the presence of the MoFe protein of nitrogenase. Although they did not report \( g \) values, Zumft and Castillo (38) showed similar spectra for, nitrogen-fixing and ammonia switched-off *R. palustris* whole cells. Their preparations were made under Ar without sodium dithionite and contain additional peaks in the \( g_{4.5} \) to 3.5 region which we eliminated by reducing the sample with sodium dithionite in the dark. We typically observed two peaks of equal amplitude in our reduced samples of *R. palustris* and *R. viridis*. However, the line widths of both peaks in *R. viridis* samples were broader than those of other whole cell organisms which we observed and approach those previously reported for isolated iron-molybdenum cofactor (FeMoco) from the MoFe protein of *A. vinelandii* (29,2).
Figure 5. A comparison of collected nitrogenase g factors (6,8) with the theoretical values of a spin 3/2 system (21). We have also included data from this laboratory. Ac: Azotobacter chroococcum; Av: Azotobacter vinelandii; Bp: Bacillus polymyxa; Cp: Clostridium pasteurianum; Cv: Chromatium vinosum; Kp: Klebsiella pneumoniae; Rj: Rhizobium japonicum; Rp: Rhodopseudomonas palustris; Rr: Rhodospirillum rubrum; Rv: Rhodopseudomonas viridis.
Ammonia switch-off of nitrogenase activity as measured by ethylene or hydrogen production is a feature common to all Rhodospirillaceae so far examined (37). It is manifested as the ability of the bacterium to rapidly cease substrate reductions by nitrogenase in vivo in response to certain ammonia-containing compounds within a period of time too short to be accounted for by a repression mechanism. Growth conditions such as light intensity (36), carbon source (35) and, especially, nitrogen source (1,31) regulate the rate and occurrence of ammonia switch-off which has been suggested to be due to covalent modification of the nitrogenase Fe protein (18) by an unidentified enzyme (32).

Nitrogenase activity of *R. palustris* and *R. rubrum* resumes in vivo upon decreased concentration of the exogenous ammonia source (38,35). The activating mechanism in vivo is unknown but is presumably the same as that observed in vitro where reactivation of isolated, inactive nitrogenase is induced by a membrane-bound activating enzyme (17). The activating enzyme has been isolated from chromatophores by salt elution (24,18) and is highly O₂-labile (17) having a half-life in air of 2 min (39). It is presumably proteinaceous due to its inactivation by trypsin (17,24). In vitro, activating enzyme binds ATP and divalent metal ions but does not hydrolyze ATP in order to activate reduced Fe protein (19). It may be stabilized in the presence of Mn²⁺ (39), which appears to be an essential requirement for growth of *R. rubrum* and *Rhodopseudomonas capsulata* under nitrogen-fixing conditions (34). Inactive Fe protein differs from active Fe protein by the presence of a covalently bound group which contains pentose, phosphate, and an adenine-like moiety on one of its two subunits (13,20). These properties set apart the nitrogenase Fe protein of the Rhodospirillaceae
from the analogous protein in the *Azotobacter*, *Klebsiella*, and *Clostridium* systems.

Upon the anaerobic addition of 0.20 mM NH₄Cl to cells growing on N₂ in the light, *R. viridis* completely stopped reducing acetylene within 5 min. However, unlike other Rhodospirillaceae, *R. viridis* nitrogenase activity did not reappear for nearly 4 h. This pattern was the same for NH₄Cl concentrations up to 0.10 M. As expected, cultures treated with 0.20 or 0.50 M NH₄Cl were still inactive after 4 h. The extended period of inactivity, regardless of NH₄Cl concentration, implies that nitrogenase reactivation may require protein synthesis of possibly the activating enzyme (if it exists in *R. viridis*) or the nitrogenase components themselves. It is not unreasonable to suspect that the activating mechanism, which is housed in the intracytoplasmic membranes of those organisms studied thus far, may differ in this organism considering the significant differences in the photosynthetic apparatus between *R. viridis* and the other Rhodospirillaceae. *R. viridis* utilizes BCHl b instead of BCHl a in its reaction centers (28) and contains intracytoplasmic membranes with a highly organized crystalline structure (12). It is also possible that, unlike *R. palustris*, the threshold level of NH₄⁺ or some intermediate metabolite which controls ammonia switch-off is not reached until 4 h following the addition of NH₄Cl.
LITERATURE CITED


PART II

**IN VIVO INTERACTION BETWEEN NITROGENASE MOLYBDENUM-IRON PROTEIN AND MEMBRANE IN AZOTOBACTER VINELANDII AND RHODOSPIRILLUM RUBRUM AS OBSERVED BY ELECTRON SPIN RESONANCE SPECTROSCOPY**
INTRODUCTION

Nitrogen fixation occurs as a result of the concerted interaction of two proteins, a molybdenum-iron protein (MoFe protein), which contains the catalytic site(s) for reduction of several substrates, including H⁺, N₂, and acetylene (C₂H₂), and an iron protein (Fe protein), which supplies electrons to the MoFe protein for use during catalysis. The properties of these proteins are relatively well conserved among the nitrogen-fixing organisms which have been examined, although the Fe protein of several Rhodospirillaceae can exist in a modified, inactive form (23) unlike the Fe proteins from Azotobacter, Clostridium, or Klebsiella. Both proteins are generally isolated from the cytoplasmic fraction of cells and, clearly, are not intrinsic membrane proteins; however, evidence does exist for functional and structural associations between the nitrogenase components and cell membrane or membrane-bound proteins.

Several types of functional associations have been observed. Nitrogenase components are highly O₂-labile, yet some organisms contain nitrogenase under aerobic or microaerophilic conditions which can be active or reversibly inactivated (protected). This is possible within variable limits of O₂ concentration dependent on the bacterium under study due to the presence of O₂-scavenging mechanisms in these organisms, the most common being respiration which occurs at the membrane (36,47,14). Several investigators have found that nitrogenase activity in vivo is enhanced by the presence of an intact, energized membrane which aids the thermodynamically unfavorable reduction of flavodoxin semiquinone from NAD(P)H, providing reducing power in a form transferrable to the Fe protein (9,44,19,20,13). Finally, the modified,
inactive Fe protein in one intensively studied member of the Rhodospirillaceae, *Rhodospirillum rubrum*, is activated in vitro and perhaps in vivo by an activating enzyme which is bound to intracytoplasmic membrane (27,24,8). Several investigators have suggested that the Fe protein may be associated with membrane via the activating enzyme during in vivo catalysis (27). Each of these lines of evidence suggests a probable functional relationship between physiological nitrogen fixation and cell membrane.

Structural associations between nitrogenase components and cell membrane have not been conclusively demonstrated. However, as much as 50% of the nitrogenase activity can remain with the membrane-containing pellet during centrifugation of an osmotic lysate of *Azotobacter vinelandii* (2). Chromatophore preparations of osmotically lysed *R. rubrum*, grown on glutamate for nitrogenase derepression, reportedly contain MoFe protein (24). Furthermore, in 1973, Stasny, Burns and Hardy (39) reported the results of immuno-ferritin labeling of the MoFe protein in thin sections of *A. vinelandii* observed by electron microscopy. The anti-MoFe protein IgG was prepared using highly purified, crystalline MoFe protein (Stasny, personal communication). Eighty percent of the immuno-label was found in the periphery of the cell cytoplasm in intimate association with the cell wall, indicating MoFe protein is discreetly localized within the cells (39). We have extended the evidence for structural interaction between the MoFe protein and cell membrane in *A. vinelandii* and *R. rubrum* through the use of electron spin resonance (esr) spectroscopy.

Esr spectroscopy has been a valuable tool in studying spatial and electronic relationships between paramagnetic molecules and membranes.
This is particularly true in biological electron transport mechanisms such as green plant or bacterial photosynthesis (3,11,10,42) and respiratory electron transport (43,33,29) for which the body of literature is quite large. Typically, membrane-bound molecules have been investigated in these studies by esr following their spatial orientation as a result of orienting the respective membranes of whole cells, isolated membrane fractions, or artificial membrane systems which carry the molecules of interest. Nitrogenase MoFe protein has well-defined esr characteristics in randomly ordered whole cell and highly purified protein preparations (2). In this study, we have observed orientation effects on the low field portion of the MoFe protein spectrum upon orienting the membranes of whole cells of _A. vinelandii_ and _R. rubrum._

In summary, we have defined our conditions for obtaining whole cell MoFe protein esr spectra which lack interfering signals in the _g_4.3 and _g_3.6 regions. Membrane systems of whole cells were oriented in two dimensions by preparing whole cell multilayers, WCM's, by methods similar to that used to produce membrane multilayers, MM's, (11). The degree of orientation of membrane-bound marker molecules, 5-doxyl stearate in _A. vinelandii_ and the bacteriochlorophyll a dimer triplet, (BChl a)_2^T_, in _R. rubrum_, was compared in WCM's to that obtained in MM's. Finally, we observed and characterized orientation dependent effects on the MoFe protein esr spectra in WCM's of nitrogen-fixing organisms. The angular dependent patterns of _R. rubrum_ and _A. vinelandii_ show several differences; however, the data suggest that a structural relationship does exist in vivo between the MoFe protein and cell membrane in these bacteria. These data were interpreted in light
of several complexities of the system including the fact that we are dealing with a double paramagnetic site (48) in an extrinsic protein-membrane association. (Portions of this work were reported in the Abstracts of the XIII International Congress of Microbiology, August, 1982, Boston).
MATERIALS AND METHODS

Organisms and growth conditions. Azotobacter vinelandii (ATCC 13705) was grown to mid-logarithmic stage in 200 ml batch cultures in modified Burk's nitrogen-free medium (40) at 30°C with shaking at 180 rpm in 1 liter culture flasks. Occasionally, Azotobacter was grown in 15 liter cultures at 30°C with air rapidly sparging through the modified Burk's medium, harvested by centrifugation in a Sharples or a Sorvall TZ-28 continuous-flow apparatus, and stored under liquid nitrogen. Fresh and frozen cells gave equivalent results in the orientation experiments.

Rhodospirillum rubrum (ATCC 17031) was grown anaerobically in 200 ml batch cultures in the medium of Ormerod, Ormerod, and Gest (30) at 30°C under light of ca. 6 mW/cm² intensity. These conditions were used to obtain cells for (BChl a)₂⁺ studies in WCM and MM preparations. Cells to be used for MM samples were often stored at -4°C prior to the preparation of chromatophores. For both (BChl a)₂⁺ and MoFe protein analyses in WCM preparations, R. rubrum was grown in the same medium with the ammonium sulfate removed in order to derepress nitrogenase, and 0.005% yeast extract added to provide enough fixed-nitrogen for the culture to begin growing. These nitrogen-fixing cultures were grown photosynthetically at 30°C in 200 ml volumes which were sparged with 95% N₂ and 5% CO₂. R. rubrum cells were grown to mid- or late-logarithmic phase, harvested anaerobically under Ar, and used immediately for WCM preparations.

Random whole cell esr samples. Whole cell esr samples of A. vinelandii were prepared similarly to the method of Davis et al. (2) in order to observe MoFe protein signals at g4.3 and g3.6. However, we
varied the redox potentials of our samples by subjecting them to the following variations: (1). Whole cells were harvested by centrifugation at 10,000 x g for 10 min; pelleted cells were transferred under air to esr tubes, capped, and frozen in liquid nitrogen. (2). Following centrifugation under air, the pelleted cells were degassed with Ar and maintained under Ar through the rest of the above procedure. (3). Following centrifugation under air, the cells were degassed with Ar and resuspended in Ar-flushed 5.0 mM phosphate buffer containing 0.10 mM methyl-viologen and 5.0 mM sodium dithionite (Na₂S₂O₄). The cells were again pelleted under Ar and transferred to Ar-flushed esr tubes, capped, and frozen as above. All samples were stored in liquid nitrogen prior to observation in the esr spectrometer.

Randomly oriented whole cells of _R. rubrum_ samples were used to determine the best conditions for obtaining MoFe protein esr signals from this organism. Freshly grown, nitrogen-fixing cells were harvested by centrifugation at 10,000 x g for 8 min under Ar in the dark. Cells were held in darkness or dim light from this point until frozen. Following centrifugation, cells were suspended in an equal volume of 40 mM Na₂S₂O₄, 0.10 mM methyl-viologen in Ar-flushed 0.10 M glycylglycine buffer. The suspension was then transferred to Ar-flushed esr tubes, capped, and frozen in liquid nitrogen where they were held until analyzed by esr spectroscopy.

**Membrane multilayers.** Chromatophores of _R. rubrum_ were prepared as previously described (6,11) by sonic disruption with a Branson sonifier (Model 200) and differential centrifugation. The chromatophore fraction was reduced with Na₂S₂O₄ prior to forming MM's (11 and Fig. 1) in order to observe the \((8\text{Chl a})_2^\top\) signal. Preparation of MM's required ca. 16
Figure 1. Preparation of membrane multilayers. Isolated membrane vesicles, shown in this figure, or whole bacterial cells were oriented by carefully collapsing them down onto a flat quartz surface. Collapse occurred as the membrane or cell suspension dried and resulted in two dimensional orientation of the membrane-bound molecules ( ), wherein two axes become parallel with the membrane surface and one axis is held perpendicular to the membrane surface. The orientation is only two dimensional, however, since the two axes parallel to the membrane plane are free to rotate about the third axis. Care was taken to prevent overdrying which destroys membrane integrity. Samples oriented by this technique, i.e. those without nitrogenase, were dried under Ar at 81% humidity by being held over saturated (NH₄)₂SO₄ for 16 to 20 h at room temperature.
Figure 1. Preparation of membrane multilayers.
to 20 h at room temperature in an 81% humidity environment.

A procedure identical to the isolation of chromatophores was used to obtain a vesicular membrane fraction from *A. vinelandii*. The membrane fraction was resuspended in an equal volume of 50 mM glycylglycine buffer. When viewed by electron microscopy, negative stains of the membrane fraction showed vesicles of nearly uniform size with few other contaminating particles. The membranes were spin labeled by adding 10 μl of freshly prepared 10 mM 5-doxyl stearate (2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidin-oxyl) in 95% ethanol to 0.5 ml of membrane suspension. The entire suspension was then used to prepare MM's as above.

**Whole cell multilayers.** In order to observe the $(BChl \ a)_2^T$ signal in non-nitrogen-fixing *R. rubrum*, approximately 0.5 ml of a thick suspension of whole cells was reduced with $Na_2S_2O_4$, layered onto quartz slides, and dried under controlled conditions as described for MM's. Similarly, whole cells of *A. vinelandii* were labeled with 5-doxyl stearate, layered and dried by a method identical to that used to produce MM's of *A. vinelandii*.

The production of WCM's of nitrogen-fixing organisms required several modifications to the above procedure in order to observe MoFe protein esr signals. Whole cells of freshly grown *R. rubrum* at mid- to late-logarithmic growth phase were harvested under Ar by centrifugation at 10,000 x g for 8 min. During this time the cells were dark adapted for approximately 20 min and kept in dim light until just before esr analysis. The supernatant was decanted from the pellet under $N_2$ (all $N_2$ used was zero grade) and added to a slightly less than equal volume of the following buffer: $N_2$-flushed 0.10 M glycylglycine (initial pH 10),
0.10 mM methyl-viologen, and freshly added 40 mM Na$_2$S$_2$O$_4$ (final pH 8). The pellet was resuspended and homogenized in buffer under N$_2$ and layered onto a quartz slide inside a custom-made glass, anaerobic chamber which was continually flushed with N$_2$. Approximately 1 h at room temperature was required to form WCM's on the slides. Samples were examined in the light at this time to assure that they were not overdried, at which point they would turn from an opaque to more translucent appearance and begin to crack or flake off the slide. *A. vinelandii* whole cells were treated in an identical manner except that it was not necessary to protect them from light. Samples were removed from the anaerobic chamber under positive N$_2$ pressure and inserted immediately into the liquid He dewar in the esr spectrometer sample cavity.

**Electron spin resonance spectroscopy.** All samples were observed in a Varian E-109 ES esr spectrometer equipped, when necessary, with a Heli-Tran liquid He cryostat (Air Products, Model LTD-3-110) and a calibrated goniometer stage for sample orientation. Light minus dark (BChl a)$_2$ spectral spectra were generated as previously described (11).

**Chemicals.** The growth media were prepared with reagent grade salts, Bacto yeast extract (Difco Laboratories), DL-malic acid and biotin (Sigma), and ethylenediaminetetraacetic acid (Fisher). Buffer components included reagent grade salts, glycylglycine (Sigma), methyl-viologen (Mann Research Laboratories, Inc.), and sodium dithionite (Sigma). The membrane spin label was 5-doxyl stearate (Syva).
RESULTS

Variations in whole cell MoFe protein esr signals. Conditions for obtaining strong, low field esr signals from nitrogenase MoFe protein were determined in randomly oriented, whole cell samples. Figure 2 shows the effects of lowering the redox potential on the $g_{4.3}$ and $g_{3.6}$ signals of the MoFe protein in A. vinelandii whole cells. Samples were prepared in the presence or absence of air and in the presence or absence of 5mM sodium dithionite (Na$_2$S$_2$O$_4$). For unexplained reasons, we found it necessary to reduce our whole cell systems in order to obtain esr spectra similar to those obtained under Ar without Na$_2$S$_2$O$_4$ as described by other investigators (Orme-Johnson, personal communication). We obtained identical spectra under our conditions for both whole cells and spheroplasts (data not shown) of A. vinelandii. As a result, Na$_2$S$_2$O$_4$ was used in all preparations of A. vinelandii to observe the MoFe protein spectra.

Whole cells of R. rubrum were prepared for esr spectroscopy in the presence of 20mM (final concentration) Na$_2$S$_2$O$_4$ following dark adaptation for approximately 20 min. This protocol served two purposes: first, it removed light-induced and oxidized, high-spin rhombic iron esr signals in the $g_{4.3}$ to 3.5 region which would interfere with the low field MoFe protein spectrum (1,38) and, second, it allowed the generation of (BChl a)$_2$ in the photosynthetic primary donor units upon illumination of the specimen while in the esr sample cavity (4,21,41). As a result, the MoFe protein and the (BChl a)$_2$ esr signals could be observed in the same cell sample. The MoFe protein spectrum obtained by this technique compared well with the esr spectrum of isolated Na$_2$S$_2$O$_4$-reduced R. rubrum MoFe protein (D.C. Yoch, personal communication).
Figure 2. Variations in whole cell *A. vinelandii* esr signals. *A. vinelandii* whole cells or spheroplasts were prepared for MoFe protein esr analysis in non-oriented samples as described in the Materials and Methods section. The redox potential of the samples decreased from (A) to (C) resulting in obvious changes in the $g=4.3$ to 3.6 region which prompted us to use Ar and Na$_2$S$_2$O$_4$ in all subsequent preparations containing nitrogenase MoFe protein. Spectrometer settings for A, B, and C: power 10mW; frequency 9.035 GHz; modulation amplitude 12.5G; time constant 0.25 s (A and B), 1 s (C); temperature 6K; gain $5 \times 10^3$ (A and B), $1.25 \times 10^4$ (C).
Membrane orientation in whole cell multilayers. Nitrogenase components are generally isolated from the cytoplasmic fraction of disrupted cells. In order to determine the existence of possible in vivo nitrogenase-membrane associations, we attempted to meet two criteria: (1) to utilize whole cells whose membrane integrity and cytoplasmic organization had been perturbed as little as possible and (2) in an attempt to orient nitrogenase MoFe protein, to orient the intracytoplasmic membranes of these organisms which undoubtedly comprise the major portion of membrane surface area available to cytoplasmic molecules.

Magnetic and flow orientation methods have been described which meet these criteria. However, these techniques can only be used for whole cells containing lamellar intracytoplasmic membrane systems that are parallel to the longitudinal axis of the rod-shaped organisms, i.e. for Rhodopseudomonas palustris and Rhodopseudomonas viridis (26,5). An alternative method for orienting intracytoplasmic membranes like those found in R. rubrum and A. vinelandii (28,32) involves isolating a membrane vesicle fraction which is then oriented into MM's (11). We therefore attempted to develop a technique for orienting R. rubrum and A. vinelandii whole cells and their intracytoplasmic membranes without rupturing the cells. Orientation of the cellular membrane systems obtained by this technique, WCM's, was compared to that obtainable with MM's by observing the orientation dependent effects induced by each method on intrinsically membrane-bound paramagnetic centers.

In R. rubrum, the bacteriochlorophyll a dimer is bound to intrinsic membrane proteins which are housed predominantly in the intracytoplasmic membranes of this organism (15,17). Previous work on isolated
intracytoplasmic membrane vesicles from _R. rubrum_ has shown that by orienting these vesicles (also called chromatophores) by MM techniques, the \((\text{BChl a})_2\) esr signal shows orientation dependent effects (11). Recently, we have been able to improve the _R. rubrum_ MM preparations to yield the complete loss of portions of the \((\text{BChl a})_2\) signal upon appropriate orientation of the MM's in the esr sample cavity. In Figures 3 and 4, we have compared the orientation dependent characteristics of the \((\text{BChl a})_2\) signal obtained from _R. rubrum_ MM and WCM preparations. The anisotropic properties of the \((\text{BChl a})_2\) signal are similar in the two samples, although the signals do not decrease to zero upon rotation of the WCM in the esr cavity. However, the signal changes in the WCM sample are comparable to previously reported _R. rubrum_ \((\text{BChl a})_2\) signal changes in MM samples (11). This indicates that a relatively high degree of membrane orientation was achieved in _R. rubrum_ WCM's and, more importantly, that the intracytoplasmic membranes were oriented by the technique.

The paramagnetic spin label, 5-doxyl stearate, was used to determine membrane orientation in MM and WCM preparations of _A. vinelandii_. Nitroxide spin labels, such as 5-doxyl stearate, have been used extensively as probes in synthetic and physiological MM specimens where their spectra are affected by numerous physical parameters including temperature, hydration, and orientation (22,16,18). The spectra of spin labeled MM's and WCM's are compared in Figures 5 and 6. Both samples showed orientation dependent effects upon 90° rotation with respect to the magnetic field, although the spectra of the label are not identical in both samples. This implies, as anticipated, that the local environment of the label is different in the two samples.
Figure 3. Orientation dependent \((B\text{Chl } a)_{2}^{T}\) spectra in \textit{R. rubrum} MM's. These two spectra of the same sample rotated 90° demonstrate essentially complete loss of portions of the triplet spectrum upon 90° rotation of the \((B\text{Chl } a)_{2}^{T}\) with respect to the magnetic field. Typical of triplet spectra, the \((B\text{Chl } a)_{2}^{T}\) is composed of six lines or three doublets centered about \(g_{2.00}\). Each doublet corresponds to absorptions by one of the three principal axes of the paramagnetic site in the \((B\text{Chl } a)_{2}^{T}\) and are arbitrarily labeled X, Y, and Z. The signal at \(g_{2.00}\) is attributed to a small concentration of bacteriochlorophyll a cation contaminant in the MM sample. (A) The normal to the membrane planes is parallel with the magnetic field direction. (B) The normal to the membrane planes is perpendicular to the field. Spectrometer settings: power 1 mW; frequency 8.981 GHz; modulation amplitude 12.5 G; time constant 0.25 s; temperature 8K; gain 6.3 \(\times 10^{-4}\).
Figure 3. Orientation dependent \((\text{Bch}l \ a)_2^T\) spectra in \textit{R. rubrum}\n
MM's.
Figure 4. Orientation dependent \((\text{BChl a})_2^T\) spectra in \textit{R. rubrum} WCM's. These spectra demonstrate the high degree of orientation obtainable by the WCM technique. All peaks are identified in the legend to Figure 3. Although none of the signals decrease to zero upon rotation of the sample by 90°, the anisotropic properties of the spectra are significant and similar to those observed in MM's. (A) The normal to the membrane planes is parallel with the magnetic field direction. (B) The normal to the membrane planes is perpendicular to the field. Spectrometer settings: power 1 mW; frequency 8.988 GHz; modulation amplitude 12.5 G; time constant 0.25 s; temperature 6K; gain $1.25 \times 10^4$. 
Figure 4. Orientation dependent $(\text{Bchl a})_2^T$ spectra in $R$. rubrum WCM's.
Figure 5. Orientation dependent 5-doxyl stearate spectra in *A. vinelandii* MM's. A membrane fraction of *A. vinelandii* prepared in the same manner as chromatophore membranes from *R. rubrum* were labeled with 5-doxyl stearate. When observed by esr spectroscopy, orientation dependent characteristics (arrows) were apparent in the spectra upon 90° rotation of the MM. (A) The normal to the membrane planes is perpendicular to the magnetic field direction. (B) The normal to the membrane planes is parallel with the field. Spectrometer settings: power 5 mW; frequency 9.047 GHz (A), 9.032 GHz (B); modulation amplitude 5 G; time constant 0.5 s; room temperature; gain 10 x 10^3.
Figure 6. Orientation dependent 5-doxyl stearate spectra in *A. vinelandii* WCM's. The 5-doxyl stearate label was immobilized in the presence of *A. vinelandii* whole cells, however, the spectra do not have identical characteristics to those in MM's. Orientation effects are apparent (arrows) upon 90° rotation. (A) The normal to the membrane planes is perpendicular to the magnetic field direction. (B) The normal to the membrane planes is parallel to the field. Spectrometer settings: power 5 mW; frequency 9.030 GHz; modulation amplitude 10G; time constant 0.128 s; room temperature; gain 2.0 x 10³.
However, these data indicate that the whole \textit{A. vinelandii} cells did collapse into WCM's and, therefore, we expected that the intracytoplasmic membranes also became oriented in these samples as had been observed for \textit{R. rubrum}.

**Orientation dependence of nitrogenase MoFe protein esr signals.**

The low field esr signals of MoFe protein, $g_{4.3}$ and $g_{3.6}$, changed in amplitude when \textit{R. rubrum} WCM's were rotated through 90° in the sample chamber (Fig. 7). By convention, at 90° the normal to the planes of the membrane surfaces in the WCM's were positioned perpendicular with the direction of the spectrometer's magnetic field and at 0° the normal to the membrane planes were parallel to the magnetic field. In order to characterize the angular dependent properties of the $g_{4.3}$ and $g_{3.6}$ signals, spectra were recorded randomly at 10° intervals from 0° to 90°. Since orientation in these samples is only achieved in two dimensions, rotating the WCM's from 90° to 180° yields the mirror image results of 0° to 90° and, therefore, was not done. In Figure 7, the amplitudes of the two \textit{R. rubrum} MoFe protein peaks were plotted as the percent of the maximum amplitude obtained. Maxima and minima occurred at 80° and 10°, respectively, for both signals. However, the angular dependent change in peak amplitude was much greater for the $g_{4.3}$ signal than for the $g_{3.6}$.

WCM's of \textit{A. vinelandii} also showed angular dependent effects on the MoFe protein signal amplitudes, however, the effects were significantly different than those observed for \textit{R. rubrum}. Throughout the series of \textit{A. vinelandii} WCM's observed, the angular dependent properties of the $g_{3.6}$ signal were constant. Figure 8 shows that the maximum amplitude occurred at 90° and the minimum at 0°, with gradual changes in amplitude
Figure 7. Angular dependence of nitrogenase MoFe protein esr signals in *Rhodospirillum rubrum* WCM's. Peak amplitudes of the $g=4.3$ and $g=3.6$ signals were plotted as percent of the maximum amplitude obtained (at 80° for each). The data represent the average of four series of angular dependent analyses performed on three individual samples. Instrumental parameters varied insignificantly through each series of spectra recorded.
Figure 8. Angular dependence of nitrogenase MoFe protein $g_{3.6}$ signal in *Azotobacter vinelandii* WCM's. Peak amplitude of the $g_{3.6}$ signal was plotted as the percent of maximum obtained ($90^\circ$). The data represent the average of four series of angular dependent analyses performed on three individual samples. Instrumental parameters varied insignificantly through each series of spectra recorded.
as the samples were rotated. However, the characteristics of the g4.3 peak in *A. vinelandii* were not consistent from one sample to the next. As shown in Figure 9, amplitude changes did not always occur in the g4.3 signal upon rotation from 0° to 90°. In other instances, the amplitude increased or decreased upon rotation.
Figure 9. Variations in the angular dependent properties of the nitrogenase MoFe protein $g_{4.3}$ signal in A. vinelandii WCM's. These three spectra were recorded from three individual WCM's which were prepared on different days from different A. vinelandii cultures. The normal to the membrane plane was oriented perpendicular (90°) to the direction of the magnetic field for the solid line spectra and parallel (0°) for the dashed line spectra. Note that the peak amplitude of the $g_{3.6}$ signal is consistently greater at 90° than at 0° while the $g_{4.6}$ peak varies in its characteristics. Spectrometer settings: power 5 mW; frequency 8.98 GHz; modulation amplitude 12.5 G; time constant 0.25 s; temperature 3.9K; gain $5 \times 10^3$ (A), $3.2 \times 10^3$ (B and C).
Figure 9.

$g = 4.3$

A. VINELANDII WCM

$g = 3.6$

$100 \text{ g}$
DISCUSSION

Changing the redox potential of whole cell and spheroplast A. vinelandii esr samples (air, Ar, or Ar+ Na₂S₂O₄) altered the appearance of the esr spectrum in the g4.3 to 3.6 region. Although other molecular species may contribute to this spectral region in whole cells, the changes we observed correlate with those induced by air or ferricyanide oxidation of isolated MoFe protein (31). However, we cannot explain why the signals produced previously in whole A. vinelandii cells under Ar (Orme-Johnson, personal communication) could only be obtained under Ar in the presence of Na₂S₂O₄ in our hands. Contaminating O₂ in the Ar may have contributed to this phenomenon or it may have been related to strain differences in the A. vinelandii cultures used.

The addition of Na₂S₂O₄ to whole cells did not reduce the MoFe protein to an esr-silent state in spite of the presence of physiological ATP sources. It is possible that the ATP concentrations were decreased in the cells by loss of O₂ in the presence of Na₂S₂O₄ and, thus, decreased oxidative phosphorylation in A. vinelandii and dark adaptation resulting in lack of photophosphorylation in R. rubrum. Lowering the ATP concentration would prevent nitrogenase activity using Na₂S₂O₄ as reductant and hold the MoFe protein at an esr-visible redox state.

The extent of membrane orientation in WCM's was nearly comparable to that obtained in MM's as evidenced by the (BChl a)₂º and 5-doxyl stearate labels. During the preparation of MM's, the isolated membrane vesicles collapse on one another to produce the multilaminar structure described in Figure 1. Although the cell walls of bacteria contain materials which lend rigidity, such as the peptidoglycan layer, whole cells commonly collapse during air drying as observed by electron
microscopy. However, the more rigid cell walls are probably less likely to collapse into smooth layers than the more flexible membrane vesicles, resulting in physical inconsistencies in the multilayer planes. This probably contributed to the observation that the \((\text{BChl a})_2^T\) signals fell to zero in MM's but not in WCM's and to the partially random nature of the 5-doxyl stearate and the MoFe protein spectra in WCM's.

The 5-doxyl stearate spectra differed slightly in the _A. vinelandii_ MM's and WCM's. The location of the spin label in whole cells was not determined; however, the label was definitely immobilized in the presence of whole cells presumably by absorption into one or more hydrophobic regions of the cell wall or cell membranes. The orientation effects on the 5-doxyl stearate spectra were less striking in the _A. vinelandii_ MM's and WCM's than those observed in pure lecithin multilayers (22). We have found this to be typical of physiological membrane systems in which the order of the spin labeled membranes is presumably perturbed by the presence of protein. The spectra of 5-doxyl stearate in _R. rubrum_ chromatophore MM's (data not shown) appear very much like those from _A. vinelandii_, while the \((\text{BChl a})_2^T\) shows strong orientation effects in the same _R. rubrum_ samples. In these systems, we find that the spectral peaks of 5-doxyl stearate do not shift as the sample is rotated, but they do change in amplitude indicating orientation in the sample.

The \((\text{BChl a})_2^T\) spectra demonstrated that the intracytoplasmic membranes of _R. rubrum_ whole cells became oriented in two dimensions by the WCM technique. Although the mechanism by which this occurred is unknown, it may involve the collapse of the intracytoplasmic membranes against the cell wall envelope during WCM formation. However,
considering the small size of the vesicular structures, 70-100 nm in diameter (15), they may rupture and flatten as the cells collapse resulting in some loss of structural interaction with cytoplasmic molecules such as the MoFe protein. This may be a second factor contributing to the partially random appearance of the MoFe protein spectra in WCM's of both organisms.

Membrane vesicle preparations of A. vinelandii yielded MoFe protein esr signals in non-oriented samples when prepared under Ar in 1 mM Na$_2$S$_2$O$_4$ (data not shown). However, due to the difficulty of isolating the membrane fraction and producing MM's in which the MoFe protein was still active, we chose to look first for orientation of MoFe protein in WCM's. In order to prepare WCM's of nitrogen-fixing cells, it was necessary to develop a technique which would give greater protection against O$_2$ damage and decrease the 16 to 20 hr period required to form multilayers in an 81% humidity atmosphere. A 4-sample glass chamber was devised with minimum space around each quartz slide where cell suspensions were placed. The chamber was constantly flushed with N$_2$ in order to dry the sample down in an even manner within 1 h at room temperature. Samples could be held at any point during the drying by sparging the N$_2$ through water prior to flushing it through the chamber.

Despite the presence of Na$_2$S$_2$O$_4$ in the A. vinelandii WCM's, the spectra obtained from these samples often appeared to be generated by a very slightly oxidized (or perhaps degraded) MoFe protein. Although we originally thought this was a result of the WCM technique, we found that R. rubrum typically did not have an enlarged g4.3 peak when prepared in the same manner. We had anticipated that R. rubrum MoFe protein would be more susceptible to oxidation during the procedure than that of A.
vinelandii, but this was not the case. Thus, the oxidized appearance of the A. vinelandii spectra may have resulted from a degradative mechanism which was not present or not operative in R. rubrum.

The MoFe protein of nitrogenase showed orientation dependent esr characteristics in WCM's of A. vinelandii and R. rubrum. Since we have shown that the cell membranes of these organisms are physically oriented in two dimensions in these samples and since the nitrogenase proteins are not intrinsic membrane proteins, but rather predominantly cytoplasmic, we suggest that the MoFe protein in these samples became oriented as the result of an extrinsic structural association with the cytoplasmic or intracytoplasmic membranes.

It is possible that the observed orientation effects resulted from crystalline MoFe protein, assuming crystallization could occur in the cytoplasm during WCM preparation. However, several arguments can be made against this hypothesis. (1). Crystallization of the MoFe protein within the cytoplasm may be possible, but it is unlikely that a single crystal of uniform orientation would form throughout the entire sample. More likely a multitude of microcrystals, randomly oriented, would form in the cells whose esr characteristics would resemble a powder spectrum. Microcrystals would only yield an anisotropic spectrum if their orientation within the cells was non-random, which could be expected only if they formed in discreet, ordered regions of the cytoplasm due to discreet localization of the MoFe protein as might be the case if it is associated with cell membrane. (2). In 1972, Davis et al. reported that the esr signal strength of purified MoFe protein decreases when it is observed in its crystalline form as opposed to a frozen solution. The strong MoFe protein signals obtained with our WCM
technique argues against the MoFe protein being crystallized. (3). In a recent attempt to study horse heart cytochrome \( c \) in pure lecithin MM's, we found the cytochrome \( c \) spectra had no orientation dependency. We interpreted this to mean that the cytochrome \( c \) had not become associated with the lecithin membranes, it had not crystallized out of solution during preparation of the MM's, nor did the physical collapse of the membrane vesicles result in orientation. (4). We have occasionally observed WCM's of some nitrogen-fixing organisms in which the MoFe protein spectra showed no orientation effects. This was particularly true in the photosynthetic organism, \textit{Rhodopseudomonas palustris}, and indicated that orientation was not caused simply by the WCM technique itself. On the basis of these criteria, therefore, we suggest it is unlikely that the nitrogenase MoFe protein orientation dependent characteristics are the result of crystallization.

Esr spectroscopy is one of several physical techniques in which spatial orientation of the molecular site under investigation can be examined. Once the principal axes of a region of interest have been assigned, it becomes theoretically possible to determine the orientation of the paramagnetic site with respect to the membrane surface in MM's (11). This approach has been most useful in studying electron transport systems in which spatial orientation is a determining factor in electronic interactions and the development of charge separation across a membrane. Although numerous characteristics of the MoFe protein have been investigated in several bacterial species, such as the amino acid sequence (25), x-ray crystallographic properties (45,46), and physical properties of the metal cluster in the iron-molybdenum cofactor (34,35), the paramagnetic site(s) is not defined well enough to establish a
principal axis system for it. However, it is useful to consider several aspects of the data.

The relative changes in peak amplitude differed between _A. vinelandii_ and _R. rubrum_. These characteristics could vary due to a number of complex factors. (1). It is not unreasonable to suspect that the physical relationship between MoFe protein and membrane is different in the two organisms. _R. rubrum_ generates ATP and reducing power by means of photosynthesis for nitrogen fixation, while _A. vinelandii_ generates ATP and reductant through respiration. If the MoFe protein is coupled to membrane for the purpose of gaining ATP or electrons for catalysis, then the coupling sites could certainly be different in the two organisms. (2). _R. rubrum_ intracytoplasmic membranes contain the activating enzyme which interacts with Fe protein and may ultimately affect the spatial relationship between Fe protein and MoFe protein near a membrane surface. This regulatory interaction does not exist in _A. vinelandii_ (7). (3). _A. vinelandii_ nitrogenase appears to interact with a third component, an iron-sulfur protein (37). A relationship with a third component is not known to exist for _R. rubrum_ nitrogenase. (4). Each of the spectra shown in Figures 7 and 8 indicates that the MoFe protein is not highly oriented. This is implied by the fact that the spectral peaks do not fall to zero with a sharp maximum inflection at a given angle. However, the apparent shapes of the angular dependence curves result in part from the fact that we are observing the sum effect of two signals generated by two paramagnetic centers (i.e., two iron-molybdenum cofactors) per MoFe protein (48). Thus, unless the two sites are oriented identically with respect to the membrane, we would expect the two overlapping spectra to have canceling
or enhancing effects on one another. This physical relationship may differ between \textit{R. rubrum} and \textit{A. vinelandii}.

Other complicating factors have to be considered in interpreting the angular dependent characteristics of the MoFe protein spectra. The partially random appearance of the spectra can be accounted for in a variety of ways. (1). As mentioned earlier, physical inconsistencies in the WCM preparations and possible disruption of the MoFe protein-membrane interaction during formation of the WCM's could contribute randomness to the samples. (2). If the MoFe protein is an extrinsic membrane protein, it may have a certain degree of freedom in its geometric relationship to the membrane which could allow a certain amount of deviation from an average orientation. This would contribute to a more random appearance in the angular dependent properties than would be observed for an intrinsic membrane protein which would be more restricted in its motion. In addition, while intrinsic membrane proteins seem to have the same orientation characteristics for analogous molecules in different organisms (12), extrinsic membrane proteins may not. (3). Finally, only a portion of the MoFe protein may be in association with membrane at any given time while the rest is subject to random distribution in the cytoplasm either before or during WCM formation.

The unusual variation in the g4.3 signal of \textit{A. vinelandii} is presently unexplained. The fact that the g3.6 peak shows strong orientation effects regardless of the g4.3 characteristics suggests that at least one of the undefined principle axes of the MoFe protein paramagnetic site which generates the g3.6 signal remains consistently oriented with respect to the membranes in the samples we have
observed. It is conceivable that the principal axis which generates the $g_{4.3}$ signal is free to change from one preparation to another. It is worth noting that the $g_{4.3}$ characteristics were typically consistent in sets of samples prepared from the same culture at the same time. This may mean that some unidentified cultural variation or technical variation caused the effects. In addition, however, since other paramagnetic molecules in whole cells can contribute to the $g_{4.3}$ signal, we may also be observing anisotropic effects from molecules other than the MoFe protein.
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EXAMINATION AND THESIS REPORT

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