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In vivo interaction between nitrogenase molybdenum-iron protein and membrane in *Azotobacter vinelandii* and *Rhodospirillum rubrum*

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Oriented whole cell multilayers of *Azotobacter vinelandii* and *Rhodospirillum rubrum* were analyzed by electron spin resonance (ESR) spectroscopy to detect possible structural associations between nitrogenase molybdenum-iron (MoFe) protein and cytoplasmic or intracytoplasmic membrane. Initially, protocols were designed to obtain strong molybdenum-iron protein ESR signals in whole cell samples of each organism. Then, two-dimensional orientation of whole cell membranes was demonstrated in whole cell multilayers using doxyl stearate spin label in *A. vinelandii* and the bacteriochlorophyll *a* dimer triplet signal, (BChl *a*)$_2^T$, from the intracytoplasmic membrane-bound photosynthetic apparatus of *R. rubrum*. Subsequent analysis of the low-field signals, $g = 4.3$ and $g = 3.6$, of molybdenum-iron protein in whole cell multilayers of each organism showed orientation-dependent characteristics, although the properties of each were different. Specifically, 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 about 37% of maximum in *A. vinelandii* and from maximum to about 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 about 63% of maximum in *R. rubrum*. These data suggest that the molybdenum-iron protein of nitrogenase was oriented in response to the physical orientation of cellular membranes and that a structural association may exist between this nitrogenase component and membrane in these organisms.

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 substrate, 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 [1]. Both proteins generally are isolated from the cytoplasmic fraction of cells and 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 lines of evidence suggest functional relationships between physiological nitrogen fixation and cell membrane. The highly O_2-labile

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Abbreviations: ESR, electron spin resonance, (BChl *a*)$_2^T$, bacteriochlorophyll *a* dimer triplet

nitrogenase components can be found in active or reversibly inactivated (protected) states in some organisms under aerobic or microaerophilic conditions. This is possible within variable limits of O_2 concentration, dependent on the bacterium, due to the presence of O_2-scavenging mechanisms in the organisms, the most common being respiration which occurs at the membrane [2–4]. Several investigators have found that nitrogenase activity in vivo is enhanced by the presence of an intact, energized membrane which is thought to aid in the production of reducing power [5–9]. Finally, the modified, inactive Fe protein in *Rhodospirillum rubrum* is activated in vitro and perhaps in vivo by an activating enzyme which is isolated from intracytoplasmic membrane fractions [10–12]. It has been suggested that the Fe protein may associate with membrane via the activating enzyme during in vivo catalysis [10].

Structural association between nitrogenase components and cell membrane has not been demonstrated conclusively, however, several lines of evidence suggest its possible existence. For example, as much as 50% of the nitrogenase activity can remain in the pellet of an osmotic lysate of *Azotobacter vinelandii* [13]. Similar amounts of nitrogenase have been detected in the membrane fraction of *A. vinelandii* ruptured by French pressure treatment [14]. Furthermore, chromatophore preparations of osmotically lysed *R. rubrum*, grown on glutamate for nitrogenase derepression, reportedly contain MoFe protein contaminant [11]. Immunoferritin labeling of the MoFe protein in thin sections of *A. vinelandii* showed eighty percent of the label in the periphery of the cell cytoplasm in intimate association with the cell wall, indicating that MoFe protein is localized predominantly within the cells at or near the cytoplasmic membrane [15]. It is also reasonable to expect some portion of the immuno-label to appear in the cytoplasm of thin sectioned bacteria such as *A. vinelandii* [16] and *R. rubrum*, which contain intracytoplasmic membrane invaginations, if nitrogenase components were associated with the surfaces of these structures.

In the research presented here, we provide further 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 and spatially oriented, whole cell membrane systems. ESR spectroscopy has been a valuable tool in studying spatial and electronic relationships between paramagnetic molecules and the membranes in which they are bound. In particular, the biological electron transport mechanisms in green plant or bacterial photosynthesis as well as in respiratory electron transport have been studied extensively by this technique [17–23]. Typically, membrane-bound paramagnetic molecules were investigated in these studies by ESR following their spatial orientation as a result of orienting the respective membranes of the whole cells, isolated membrane fractions, or artificial membrane systems which carried the molecules of interest. Nitrogenase MoFe protein has well-defined ESR characteristics in randomly ordered whole cell and highly purified protein preparations [13]. 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*

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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 [24] 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 filtered 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 [25] at 30°C under light of ca. 6 mW/cm^2 intensity. These conditions were used to obtain cells for (BChl a)$_2^T$ studies in whole cell multilayer and membrane multilayer preparations. Cells to be used for membrane multilayer samples were often stored

at −4°C prior to the preparation of chromatophores. When both (BCHl $a)_2^T$ and MoFe protein were analyzed in the same preparation, *R. rubrum* was grown in the above 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% N_2 and 5% CO_2. *R. rubrum* cells were grown to mid- or late-logarithmic phase, harvested anaerobically under argon, and used immediately for whole cell multilayer preparations.

Randomly oriented whole cell ESR samples. Whole cell ESR samples of *A. vinelandii* were prepared similarly to the method of Davis et al. [13] in order to observe MoFe protein signals at $g = 4.3$ and $g = 3.6$. However, the redox potentials of our samples were varied by subjecting them to the following conditions; (1) Whole cells were harvested by centrifugation at $10000 \times g$ 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 argon and maintained under argon through the rest of the above procedure. (3) Following centrifugation under air, the cells were degassed with argon and resuspended in argon-flushed 5.0 mM phosphate buffer containing 0.10 mM methyl viologen and 5.0 mM sodium dithionite ($Na_2S_2O_4$). The cells were again pelleted under argon and transferred to argon-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* were used to determine the best conditions for obtaining MoFe protein ESR signals. Freshly grown, nitrogen-fixing cells were harvested by centrifugation at $10000 \times g$ for 8 min under argon in the dark. Cells were held or dim light from this point until frozen. Following centrifugation, cells were suspended in an equal volume of 40 mM $Na_2S_2O_4$, 0.10 mM methyl viologen in argon-flushed 0.10 M glycylglycine buffer. The suspension was then transferred to argon-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 [18,26] using sonic disruption with a Branson sonifier (model 200) and differential centrifugation. The chromatophore fraction was reduced with $Na_2S_2O_4$ prior to forming membrane multilayers [18] in order to observe the (BChl $a)_2^T$ signal. Preparation of membrane multilayers required approx. 16 to 20 h at room temperature in an 81% humidity argon 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-oxazolidinyloxyl) in 95% ethanol to 0.5 ml of membrane suspension. The entire suspension was then used to prepare membrane multilayers as above.

Whole cell multilayers. In order to observe the (BChl $a)_2^T$ signal in non-nitrogen-fixing *R. rubrum,* approx. 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 membrane multilayers. Similarly, whole cells of *A. vinelandii* were labeled with 5-doxyl stearate, layered and dried as described above.

The production of whole cell multilayers 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 argon by centrifugation at $10000 \times g$ for 8 min. During this time the cells were dark adapted for approx. 20 min and kept in dim light until just before ESR analysis. The supernatant was decanted under N_2 (all N_2 used was zero grade) and a slightly less than equal volume of the following buffer was added to the pellet: N_2-flushed 0.10 M glycylglycine (initial pH 10), 0.10 mM methyl viologen, and freshly added 40 mM $Na_2S_2O_4$ (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 whole cell multilayers 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 slides. Samples could be held at any point during the drying by sparging the N_2 through water prior to flushing it through the chamber. *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 cryostat in the ESR spectrometer sample cavity.

Electron spin resonance spectroscopy. All spectra were recorded with 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 $a)_2^T$ spectra were generated as previously described [18].

Chemicals. The growth media were prepared with reagent grade salts, Bacto yeast extract (Difco Laboratories), DL-malic acid and biotin (Sigma), and ethylenediaminetetraacetic acid (Fischer). 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. Fig. 1 shows the effects of 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 5 mM sodium dithionite ($Na_2S_2O_4$). For unexplained reasons, we found it necessary to reduce our whole cell systems with $Na_2S_2O_4$ in order to obtain ESR spectra

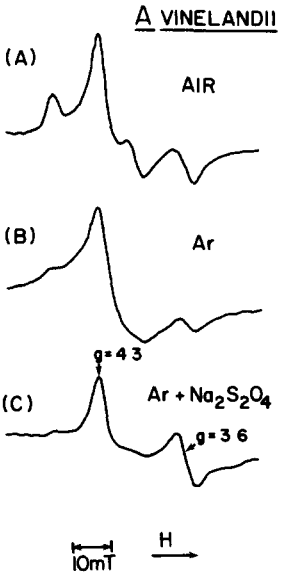

Fig 1. Variations in *A vinelandii* whole cell MoFe protein ESR signals *A vinelandii* whole cells were prepared for MoFe protein ESR analysis in non-oriented samples while altering the redox potential of the systems as described in Methods The redox potential of the samples decreased from (A) to (C) resulting in obvious changes in the $g = 4\,3$ and $g = 3.6$ peaks which prompted us to use argon and $Na_2S_2O_4$ in all subsequent preparations containing nitrogenase MoFe protein Spectrometer settings were as follows· microwave power 10 mW; frequency 9 035 GHz; modulation amplitude 1.25 mT, time constant 0 25 s (A and B), 1 s (C); temperature 6 K; gain $5 \cdot 10^3$ (A and B), $1\,25 \cdot 10^4$ (C).

similar to those obtained under argon without $Na_2S_2O_4$ as described by other investigators (Ref. 13 and Orme-Johnson, personal communication). We obtained identical spectra under our conditions for both whole cells and spheroplasts (data not shown) of *A. vinelandii*. Although other paramagnetic species may contribute to this spectral region in whole cells, the spectra we observed in unreduced samples correlate with those induced by air or ferricyanide oxidation of isolated MoFe protein [27].

Whole cells of *R. rubrum* were prepared for ESR spectroscopy in the presence of 20 mM (final concentration) $Na_2S_2O_4$ 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 $g = 4.3$–3.5 region which would interfere with

the low-field MoFe protein spectrum [28,29] and, second, it allowed the generation of (BChl a)$_2^T$ in the photosynthetic primary donor units upon illumination of the specimen while in the ESR sample cavity [30–32]. As a result, the MoFe protein and the (BChl a)$_2^T$ ESR signals could be observed in the same cell sample. The low-field MoFe protein spectrum obtained by this technique (Fig. 4) compared well with the ESR spectrum of isolated $Na_2S_2O_4$-reduced *R. rubrum* MoFe protein (Yoch, D.C., personal communication). It should be mentioned that both *R. rubrum* and *A. vinelandii* whole cells exhibit a number of resonances in the $g = 2.0$ region which obscure the detection of the third (i.e., $g = 2.0$) signal of the MoFe protein of nitrogenase.

Membrane orientation in whole cell multilayers

Nitrogenase components generally are isolated from the cytoplasmic fraction of disrupted cells. In order to determine the existence of possible in vivo nitrogenase-membrane associations, we attempted to utilize whole cells whose membrane integrity and cytoplasmic organization had been perturbed as little as possible and orient the intracytoplasmic membranes of these organisms which comprise the major portion of membrane surface area available to cytoplasmic molecules [51].

Magnetic and flow orientation methods have been described which meet these criteria for some organisms. However, these techniques can be used only for whole cells containing lamellar intracytoplasmic membrane systems, for example, *Rhodopseudomonas palustris* and *Rhodopseudomonas viridis* [33,34]. An alternative method for orienting intracytoplasmic membranes like those found in *R. rubrum* and *A. vinelandii* [35,36] involves isolating a membrane vesicle fraction which is then oriented into membrane multilayers [18]. 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 was compared to that obtained with membrane multilayers by observing the orientation-dependent effects induced by each method on intrinsically membrane-bound paramagnetic centers.

In *R. rubrum*, the primary donor bacteriochlorophyll *a* dimer is bound to intrinsic membrane proteins (i.e., the photosynthetic reaction center) which are housed predominantly in the intracytoplasmic membranes of this organism [37,38]. Photochemical excitation of the reaction center under physiological condition induces the transfer of an electron from the bacteriochlorophyll dimer to the primary acceptor, a quinone magnetically coupled to an iron atom [39]. Chemical reduction of the primary acceptor blocks this electron transfer reaction and results in the photo-excited donor eventually being converted to an excited triplet state, i.e., (BChl a)$_2^T$. The ESR spectrum of (BChl a)$_2^T$ is highly anisotropic and, therefore, depends on the orientation of the dimer in the magnetic field of the spectrometer. Previous work on isolated intracytoplasmic membrane vesicles from *R. rubrum* has shown that by orienting these vesicles (also called chromatophores) by membrane multilayer techniques, the (BChl a)$_2^T$ ESR signal shows orientation dependent effects [18]. Recently, we have been able to improve the *R. rubrum* membrane multilayer preparations to yield the complete loss of portions of the (BChl a)$_2^T$ signal upon appropriate orientation of the multilayers in the ESR sample cavity. In Fig. 2, we have compared the orientation dependent characteristics of the (BChl a)$_2^T$ signal obtained from *R. rubrum* membrane multilayers and whole cell multilayers. The anisotropic properties of the (BChl a)$_2^T$ signal are similar in the two samples, although the signal intensities do not decrease to zero upon rotation of the whole cell multilayers in the ESR cavity. However, the signal changes in the whole cell multilayer sample are comparable to previously reported *R. rubrum* (BChl a)$_2^T$ signal changes in membrane multilayer samples [18]. This indicates that a relatively high degree of membrane orientation was achieved in *R. rubrum* whole cell multilayers 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 *A. vinelandii*. Nitroxide spin labels, such as 5-doxyl stearate, have been used extensively as probes in synthetic and physiological multilayer specimens where their spectra are affected by numerous physical parameters including temperature, degree

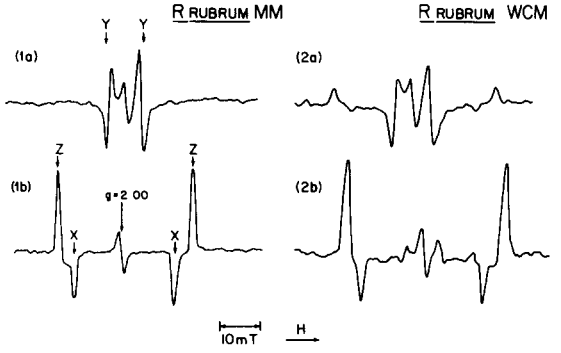

Fig. 2 Orientation-dependent (BChl a)$_2^T$ spectra in *R rubrum* membrane and whole cell multilayers. These spectra were obtained from a membrane multilayer (MM) (1) and a whole cell multilayer (WCM) (2) preparation of $Na_2S_2O_4$-reduced *R rubrum* oriented 0° and 90° in the ESR sample cavity by recording light-minus-dark (BChl a)$_2^T$ spectra Typical of triplet spectra, the (BChl a)$_2^T$ is composed of three doublets centered about $g = 2.00$ Each doublet corresponds to absorptions by one of the three principal axes of the paramagnetic site and are arbitrarily labeled X, Y, and Z. The signal at $g = 2.00$ is attributed to a small concentration of bacteriochlorophyll dimer cation radical generated in unreduced samples. Rotation of the membrane multilayer sample by 90° resulted in essentially complete loss of portions of the triplet spectrum implying a very high degree of orientation of the bacteriochlorophyll dimer Although none of the signals decreased to zero upon rotation of the whole cell multilayer, the anisotropic properties of the spectra indicate a high degree of orientation in the sample. (a) The normal to the membrane planes is parallel (0°) with the magnetic field direction. (b) The normal to the membrane planes is perpendicular (90°) to the field. Spectrometer settings were as follows microwave power 1 mW, frequency (1) 8.981 GHz, (2) 8 988 GHz, modulation amplitude 1.25 mT, time constant 0 25 s; temperature (1) 8 K, (2) 6 K, gain (1) $6\,3\cdot10^4$, (2) $1.25\cdot10^4$.

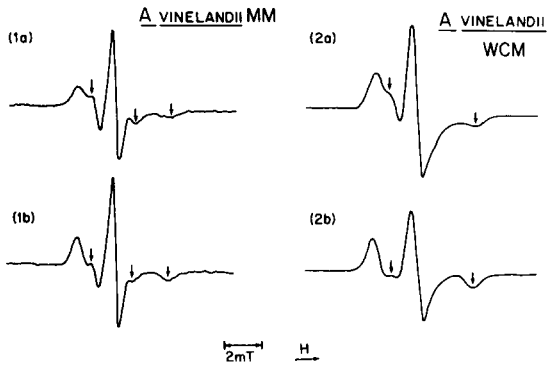

Fig. 3 Orientation dependent 5-doxyl stearate spectra in *A vinelandii* membrane multilayers (MM) (1) and whole cell multilayers (WCM) (2) Orientation dependent spectral changes (arrows) were apparent when the sample labeled with 5-doxyl stearate was rotated 90° in the ESR sample cavity However, the whole cell and membrane multilayer spectra do not have identical characteristics. (a) The normal to the membrane planes is parallel (0°) with the magnetic field direction (b) The normal to the membrane planes is perpendicular (90°) to the field Spectrometer settings were as follows microwave power 5 mW; frequency (1a) 9 047 GHz, (1b) 9 032 GHz, (2) 9 030 GHz, modulation amplitude (1) 0 5 mT, (2) 1.0 mT, time constant (1) 0.5 s, (2) 0 128 s, room temperature, gain (1) $10\ 10^3$, (2) $2\,0\cdot10^3$

of hydration, and orientation [40–42]. The spectra of spin labeled membrane and whole cell multilayers are compared in Fig. 3. 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. However, these data indicate that the whole *A. vinelandii* cells did collapse into whole cell multilayers and we anticipate that the intracytoplasmic membranes also became oriented in these samples as had been observed for *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 *R. rubrum* whole cell multilayers were rotated through 90° in the ESR sample cavity (Fig. 4). By convention, at 90° the normal to the planes of the membrane surfaces was positioned perpendicular to the direction of the spectrometer's magnetic field and at 0° the normal to the membrane planes was 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 achieved only in two dimensions, rotating from 90° to 180° yields the mirror image results of 0° to 90° and, therefore, is not shown. In Fig. 4, the amplitudes of the two *R. rubrum* MoFe protein peaks were plotted as the percent of the maximum amplitude obtained. Maxima and minima occurred at 80° and 10°,

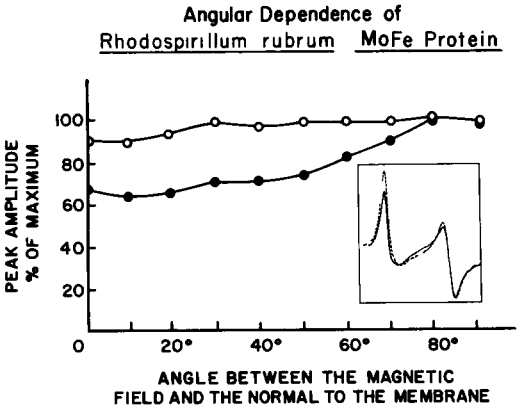

Fig 4 Angular dependence of nitrogenase MoFe protein ESR signals in *R rubrum* whole cell multilayers Peak amplitudes of the $g = 4.3$ and $g = 3.6$ signals were plotted as percent of the maximum amplitude obtained at 80° Spectra were recorded as samples were positioned at each angle in random order. The data represent the average of four series of angular-dependent analyses performed on individual samples Instrumental parameters varied insignificantly through each series of spectra recorded and were essentially the same as those reported in Fig 6 (○), $g = 3.6$, (●) $g = 4.3$ signal amplitudes Inset. Representative low-field spectra of *R rubrum* whole cell multilayer MoFe protein Solid line, 10°; dashed line, 80°

respectively, for both signals. However, the angular-dependent change in peak amplitude was much greater for the $g = 4.3$ signal than for the $g = 3.6$.

Whole cell multilayers of *A. vinelandii* also showed angular-dependent effects on the MoFe protein signal amplitudes, however, the effects were significantly different than those observed for *R. rubrum*. Throughout the series of *A. vinelandii* whole cell multilayers observed, the angular dependent properties of the $g = 3.6$ signal were constant. Fig. 5 shows that the maximum amplitude occurred at 90° and the minimum at 0°, with gradual changes in amplitude as the samples were rotated. On the other hand, the characteristics of the $g = 4.3$ peak in *A. vinelandii* were not consistent from one sample to the next. As shown in Fig. 6, amplitude changes did not always occur i

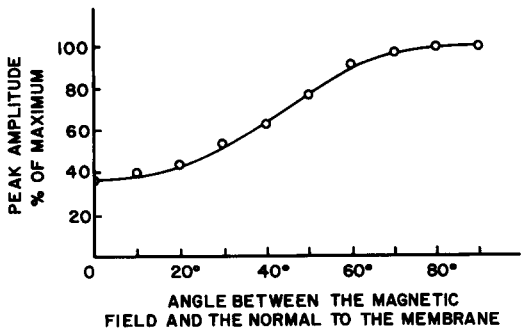

Fig. 5 Angular dependence of nitrogenase MoFe protein $g = 3.6$ signal in *A. vinelandii* whole cell multilayers. Peak amplitude of the $g = 3.6$ signal was plotted as the percent of maximum obtained at 90°. The conditions of the analyses were identical to those reported in Fig. 4.

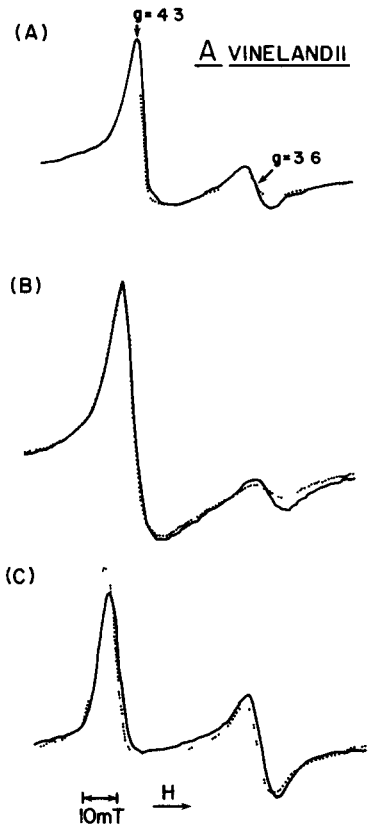

Fig. 6 Variations in the angular-dependent properties of the nitrogenase MoFe protein $g = 4.3$ signal in *A vinelandii* whole cell multilayers. These three spectra were recorded from three individual whole cell multilayers prepared on different days from different *A. vinelandii* cultures. Note that the peak amplitude of the $g = 3.6$ signal is consistantly greater at 90° than at 0° while the $g = 4.3$ peak varies in its characteristics Solid line, 90°; dashed line, 0°. Spectrometer settings were as follows: microwave power 5 mW, frequency 8.980 GHz; modulation amplitude 1.25 mT; time constant 0.25 s; temperature 6 K, gain (A) $5 \cdot 10^3$, (B, C) $3.2 \cdot 10^3$.

the $g = 4.3$ signal upon rotation from 0° to 90°. In other instances, the amplitude increased or decreased upon rotation. It is also apparent that, unlike *R. rubrum*, the $g = 4.3$ peak of *A. vinelandii* whole cell multilayers is oversized when compared to the MoFe protein signals of randomly oriented whole cells (Fig. 1C).

Discussion

During catalytic turnover, the MoFe protein of nitrogenase is reduced to an ESR-silent state. However, the addition of $Na_2S_2O_4$ to whole cells in our system did not reduce the MoFe protein to an ESR-silent state as occurs in vitro in spite of the presence of both Fe protein and physiological ATP sources. It is possible that the ATP concentrations were decreased in *A. vinelandii* by loss of O_2 in the presence of $Na_2S_2O_4$ and, thus, by loss of oxidative phosphorylation, and, in *R. rubrum* by dark adaptation resulting in lack of photophosphorylation. Lowering the ATP concentration could prevent nitrogenase from using $Na_2S_2O_4$ as reductant and, therefore, could hold the MoFe protein at an ESR-visible redox state.

The extent of membrane orientation in whole cell multilayers was nearly comparable to that obtained in membrane multilayers as evidenced by the (BChl a)$_2^T$ and 5-doxyl stearate spectra. During the preparation of membrane multilayers, the isolated membrane vesicles presumably collapse on one another to produce a multilamellar structure. 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 rigid cell walls are probably less likely to collapse into smooth layers than more flexible membrane vesicles, resulting in physical inconsistencies in the multilayer planes. This probably contributes to the observation that the (BChl a)$_2^T$ was more highly oriented in membrane multilayers than in whole cell multilayers.

The 5-doxyl stearate spectra differed slightly in the *A vinelandii* membrane and whole cell multilayers. 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 either multilayer preparation of *A. vinelandii* than those observed in pure lecithin multilayers [40]. 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. For example, the spectrum of 5-doxyl stearate in *R. rubrum* chromatophore multilayers (data not shown) appears very much like those from *A. vinelandii*, while the (BChl a)$_2^T$ shows strong orientation effects in the same *R. rubrum* samples [18]. In these systems, we find that the spectral locations of the peaks of 5-doxyl stearate do not shift as the sample is rotated but only change in amplitude to indicate orientation in the sample.

The (BChl a)$_2^T$ spectra demonstrated that the intracytoplasmic membranes of *R. rubrum* whole cells became oriented in two dimensions by the whole cell multilayer 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 multilayer formation. However, considering the small size of the vesicular structures, 70–100 nm in diameter [37], they may rupture and flatten as the cells collapse resulting in some loss of structural interaction with cytoplasmic molecules such as the MoFe protein.

Despite the presence of $Na_2S_2O_4$ in the *A vinelandii* whole cell multilayers, the spectra obtained from these samples often contained an oversized $g = 4.3$ peak possibly due to a small fraction of oxidized MoFe protein. Although we originally thought this occurred as a result of the technique, we found that *R. rubrum* typically did not have an enlarged $g = 4.3$ 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 operative in *R. rubrum* or is perhaps the result of contaminating signals other than oxidized MoFe protein in *A. vinelandii* which are not reduced at the dithionite concentrations used.

The MoFe protein of nitrogenase showed orien-

tation dependent ESR characteristics in whole cell multilayers 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 may have become 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 whole cell multilayer 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 portein as might be the case if it is associated with cell membrane. (2) Davis et al. [13] 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 multilayer technique argue against the MoFe protein being crystallized. (3) In a recent attempt to study horse heart cytochrome *c* in pure lecithin multilayers, 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 and that it had not crystallized out of solution during preparation of the multilayers, nor did the physical collapse of the membrane vesicles result in orientation. (4) We have occasionally encountered whole cell multilayers of some nitrogen-fixing organisms in which the MoFe protein spectra showed no orientation effects. This was particularly true in the photosynthetic organism *Rhodopseudomonas palustris* and implies that orientation is not caused simply by the whole cell multilayer technique itself. (5) *A. vinelandii* MoFe protein has been crystallized successfully, however, we also see orientation of *R. rubrum* MoFe protein which has never been crystallized. On the basis of these criteria, therefore, we suggest it is unlikely that the nitrogenase MoFe protein orientation is an artifact of the multilayer technique or the result of in vivo protein 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 membrane multilayers [18]. This approach has been useful in studying electron transport systems in which spatial orientation is a determining factor in electronic interactions and 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 [43], X-ray crystallographic properties [44,45], and physical properties of the metal cluster of the iron-molybdenum cofactor [46,47], 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 this data which relate to the structural relationship between the paramagnetic site(s) and the membrane.

The relative changes in peak amplitude differed between *A. vinelandii* and *R. rubrum* (Figs. 4 and 5) suggesting a different physical relationship exists between MoFe protein and membrane in the two organisms. This suggestion would not be unreasonable for the following reasons: (1) *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 is not known to exist in *A vinelandii* [48]. (3) *A. vinelandii* nitrogenase appears to interact with a third component, an iron-sulfur protein [49]. A relationship with a third component of this type is not known to exist for *R. rubrum* nitrogenase.

The nitrogenase spectra in whole cell multilayers indicates that the MoFe protein is not highly oriented. This is implied by the fact that the amplitudes of the spectral peaks do not decrease to zero or have a sharp maximum inflection point upon rotating the samples. However, this partially random appearance may be due to several factors. (1) 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 [50]. 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 causing the loss of sharp maximum and minimum points. (2) As mentioned earlier, physical inconsistencies in the whole cell multilayer preparations and possible disruption of the MoFe protein-membrane interaction during formation of the multilayers could contribute randomness to the samples. (3) If the MoFe protein is an extrinsic membrane protein, it may have a degree of freedom in its geometric relationship to the membrane which allows deviation from an average orientation. This would contribute to a more random appearance in the angular dependent properties than may be observed for an intrinsic membrane protein which may 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 (Hales, B.J., Howard, K.S. and Case, E.E., unpublished data), extrinsic membrane proteins may not. (4) 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 whole cell multilayer formation.

The unusual variation in the $g = 4.3$ signal of *A. vinelandii* is presently unexplained. The fact that the $g = 3.6$ peak shows strong orientation effects regardless of the $g = 4.3$ characteristics suggests that at least one of the undefined principal axes of the MoFe protein paramagnetic site which generates the $g = 3.6$ 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 important to note that the $g = 4.3$ characteristics were typically consistent within sets of samples prepared from the same culture at the same time. This may mean that some unidentified cultural or technical variation caused the effects. In addition, since other paramagnetic molecules in whole cells can contribute to the $g = 4.3$ signal, we may be observing anisotropic effects from molecules other than the MoFe protein in these particular *A. vinelandii* samples.

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