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Complex Formation and O_2 Sensitivity of *Azotobacter vinelandii* Nitrogenase and Its Component Proteins†

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ABSTRACT: The O_2 stability of the MoFe protein, the Fe protein, a 1:1 mixture of these proteins, and a 1:1 mixture in the presence of the *Azotobacter vinelandii* FeS-II protein has been studied as a function of time under controlled O_2 partial pressures. The Fe protein is much more sensitive to O_2 exposure than is the MoFe protein. The presence of the FeS-II protein at a 1:1 ratio with the component proteins measurably increases the O_2 stability of the MoFe and Fe proteins. O_2 inactivation of the MoFe protein was studied in some detail and found to be quite complex. At least three partially overlapping reactions are suggested. The first is the reversible oxidation of the metal clusters of the MoFe protein to the combined extent of 12 electrons with full retention of activity. The second phase consists primarily of activity loss with little increase in the extent of reversible oxidation. The third phase continues to decrease the protein activity but is also accompanied by formation of a $g = 2.0$ EPR signal and more extensive oxidation. Ultracentrifugation studies of the FeS-II protein at a 1:1:1 ratio with the Fe and MoFe proteins do not support the formation of the Bulen complex. The formation of other O_2-stable complexes is discussed.

Nitrogenase is an O_2-sensitive, two-component protein system found in several biological organisms ranging from blue-green algae and rapidly respiring aerobic bacteria, where high concentrations of O_2 are likely to be present, to microaerophylic and anaerobic bacteria, where O_2 concentrations are relatively low. The nitrogenases from all of these biological sources are remarkably similar (Mortenson & Thorneley, 1979) and all catalyze the reduction of N_2 to ammonia. How these organisms cope with the problem of protecting the nitrogenase enzyme from O_2 inactivation is of considerable interest but is still without satisfactory explanation. One approach to studying this problem is to examine the O_2 sensitivity of both component proteins comprising nitrogenase and the isolable protein complex consisting of the strongly associated MoFe protein (M_r 230 000, 30 Fe and 2 Mo), the Fe protein (M_r 65 000, 4Fe), and an iron-sulfur protein FeS-II (ferredoxin II, Shethna protein; M_r 24 000, 2Fe). There is

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general agreement that the isolated nitrogenase complex is quite stable toward O_2 inactivation (Bulen & LeComte, 1972) whereas the isolated components (especially the Fe protein) are both rapidly inactivated by O_2 exposure. The nature of the O_2-stable complex and the mechanism by which the components interact in the complex are interesting and important questions.

Studies of the isolated nitrogenase complex from *Azotobacter vinelandii* and *Azotobacter chroococcum* indicate (Haaker & Veeger, 1977; Robson, 1979) that the low molecular weight [2Fe-2S] protein, FeS-II, found associated with the complex is responsible for the O_2 stability of this form of nitrogenase. This result has been deduced from a number of studies (Haaker & Veeger, 1977; Robson, 1979; Scherings et al., 1983) which have noted that a mixture of the purified component proteins is less readily inactivated by O_2 in the presence of the FeS-II protective protein than in its absence. The conclusion has been reached that O_2 stability is imparted to the separately isolated O_2-sensitive components by the FeS-II protective protein (Haaker & Veeger, 1977) presumably by formation of a protective complex. However, this reconstituted system, though more resistant to O_2 inactivation than the isolated components, still lacks the high degree of O_2 stability that the native complex originally possessed. Further studies to disclose details of the native and reconstituted complex seem warranted.

We report here results of experiments that examine the O_2 inactivation of each of the component proteins from nitrogenase and a mixture of the two nitrogenase components with the FeS-II protective protein at a 1:1:1 ratio. The O_2-exposure studies were carried out under carefully controlled conditions and provide a self-consistent set of results to compare the relative O_2 sensitivities of the MoFe protein, the Fe protein, a 1:1 mixture of these two proteins, and this mixture in the presence of the FeS-II protective protein.

Experimental Procedures

Nitrogenase Proteins. The Fe protein with specific activity of 2400 nm of $H_2 \cdot min^{-1} \cdot mg^{-1}$ containing 3.6 Fe atoms per mole and the MoFe protein with specific activity of 2000–2500 nm of $H_2 \cdot min^{-1} \cdot mg^{-1}$ containing 25–28 Fe and 1.7–1.9 Mo atoms per mole were prepared by the method of Burgess et al. (1980) and freed from excess $S_2O_4^{2-}$ by anaerobic Sephadex G-25 chromatography. The preparation of the nitrogenase complex and its characterization have been previously described (Bulen & LeComte, 1972). The FeS-II protective protein (also referred to as Shethna II and *A. vinelandii* ferredoxin II) was prepared and characterized by a method similar to that of Robson (1979). Protein concentrations were determined by the Lowry or Biuret methods using crystalline bovine serum albumin (BSA) as a standard or from A_{400} values for the MoFe protein (Watt et al., 1981).

Standard H_2 evolution conditions previously described (Wherland et al., 1981) were used to assay protein activity. Assays were conducted under argon at 30 °C for 8 min after which sulfuric acid (0.25 mL) was used to quench the reaction. H_2 evolved under these conditions was determined by gas chromatography.

O_2 Exposure. Two types of O_2-exposure experiments were conducted. One consisted of reacting protein samples for a fixed time period of 2.5 min (1 min of vortexing, 1.5 min of stirring) at various O_2 partial pressures in argon from which activity loss as a function of O_2 partial pressure was determined. The second type evaluated activity loss from protein samples exposed to a constant O_2 partial pressure as a function of time. Most O_2 exposure experiments were conducted with O_2–argon mixtures, but some parallel experiments were carried out by using an air (20% O_2, 80% N_2)–argon mixture as a source of O_2 to determine if the presence of N_2 altered the O_2 sensitivity of the nitrogenase proteins and also to allow comparison of our results with those studies using air as a source of O_2.

The nitrogenase protein (Fe, MoFe, or a mixture of both) under study was first freed from $S_2O_4^{2-}$ by anaerobic Sephadex G-25 chromatography in a Vacuum Atmospheres glove box (<1 ppm of O_2) and verified to be fully reduced and free of $S_2O_4^{2-}$ by controlled potential coulometry; 1.0 mL was then injected into a capped 10-mL assay bottle containing a Teflon stir bar and the desired O_2 partial pressure in argon. The protein sample was vortexed for 1 min after which it was vigorously stirred and sampled for activity (in triplicate) as a function of time. Parallel reactions in the absence of O_2 showed that the vortexing and stirring had no detrimental effect on the protein activity. Reaction bottles containing 1 mL of buffer under various O_2 partial pressures were vortexed and stirred as was done for the protein solutions, and 50-μL aliquots were removed and injected into the coulometry cell as a function of time to determine the O_2 concentration in the liquid phase. These control reactions demonstrated that gas–liquid equilibrium was established in less than 15 s of vortexing and remained constant during the period of the experiment.

MoFe and Fe protein samples partially or fully inactivated by O_2 as described above were passed through anaerobic G-25 columns to remove O_2, O_2 reduction products, or any other small chemical fragments resulting from O_2 exposure. The protein was then analyzed for Fe and Mo, reduced coulometrically to measure the extent of oxidation, and characterized spectroscopically.

Ultracentrifugation. Sedimentation values at 25 °C in 0.05 M tris(hydroxymethyl)aminomethane (Tris)–0.25 M NaCl, pH 8 (in the presence and absence of 1 mM $S_2O_4^{2-}$), for the MoFe protein and Fe protein were determined separately in a Beckman Model E ultracentrifuge as a function of concentration over the range 2–16 mg/mL. These sedimentation values were used to monitor the presence of the Fe and MoFe proteins in the following protein mixtures. The 1:1 mixtures of reduced $S_2O_4^{2-}$-free Fe protein and MoFe protein in the presence and absence of 1 mM $MgCl_2$, 1 mM $S_2O_4^{2-}$, and 1 mM MgATP were examined at 48 000 rpm over a 2.5-h period. A 1:1 mixture of Fe protein and MoFe protein each previously oxidized with methylene blue by one and six electrons, respectively, was also studied. Duplicate studies to those just described, including the methylene blue oxidized proteins, were carried out except 1.10 mol of FeS-II was added per mol of MoFe protein and Fe protein. Sedimentation values of protein peaks in these mixtures were determined and compared to those of the MoFe protein, the Fe protein, and the Bulen complex determined separately under similar conditions. Following the ultracentrifugation runs, the contents of the cell were assayed and samples injected into a coulometry cell to assess the redox status of the proteins. No significant activity loss (±10%) was observed after ultracentrifugation, nor was any oxidation of reduced protein detected (the microcoulometry method used was sensitive to ±1e).

Nitrogenase complex prepared by the method of Bulen and LeComte was passed through an anaerobic Bio-Gel P-150 column equilibrated in 0.01 M 2-[[tris(hydroxymethyl)-methyl]amino]ethanesulfonic acid (TES), pH 7.0, and then loaded into the ultracentrifugation cell. Sedimentation values were determined in the 1–16 mg/mL concentration range and

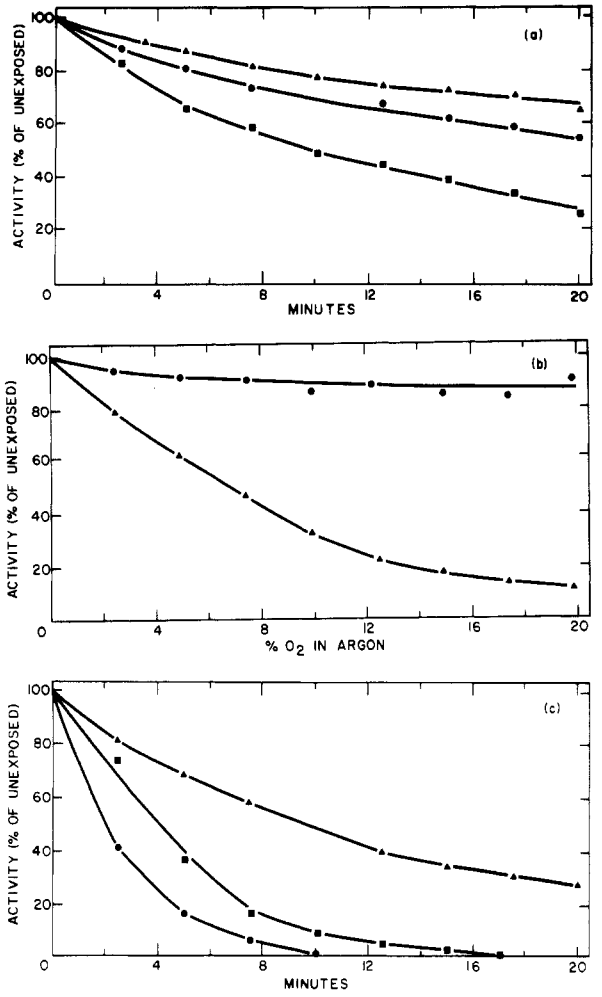

FIGURE 1: Reaction of $S_2O_4^{2-}$-free proteins with O_2 in argon. (a) Activity resulting from triplicate measurements, of MoFe protein in 0.05 M Tris–0.25 M NaCl, pH 8.0, during exposure to (▲) 5% O_2 in argon, (●) 10% O_2 in argon, and (■) 20% in argon. (b) Activity of MoFe protein (●) and Fe protein (▲) as a function of partial pressure of O_2 in argon. The activity was measured in triplicate after 1 min of vortexing followed by 1.5 min of rapid stirring. (c) Activity of Fe protein resulting from triplicate measurements of Fe protein in 0.05 M Tris–0.25 M NaCl, pH 8.0, during exposure to (▲) 2.5% O_2 in argon, (■) 5% O_2 in argon, and (●) 10% O_2 in argon. In all experiments, the protein studied was about 10 mg/mL and was diluted more than 10 times for activity measurements to minimize activity loss due to high NaCl concentration.

extrapolated to zero protein concentration.

Coulometry. A previously described (Watt, 1979) coulometric procedure was used to determine the oxidation state of the nitrogenase proteins following their exposure to O_2 and also to establish that no oxidation of reduced proteins had occurred during ultracentrifugation experiments. Because O_2 and some of its reduction products, i.e., H_2O_2, HO_2^-, and O_2^-, are redox active, it was necessary to free the O_2-exposed proteins from these small molecules in order to determine the true redox state of the protein resulting from O_2 exposure. This was done by passing the O_2-exposed protein mixture through an anaerobic Sephadex G-25 column and collecting the emerging protein band. The protein concentration was determined by Lowry or biuret methods, and samples were injected into a coulometry cell to measure the extent of protein oxidation.

Spectroscopic Measurements. A Cary 118 was used for recording optical spectra of O_2-exposed nitrogenase proteins and for following the time course of oxidation of these proteins during O_2 exposure. A Varian Model 4502 EPR spectrometer was used for recording EPR spectra of MoFe protein and Fe protein samples after reaction with O_2. Protein samples of 6–9 mg/mL were stirred under 10% and 5% O_2 in argon, respectively, and 0.25-mL samples were removed as a function of time and frozen in 3 mm i.d. quartz EPR tubes. EPR spectra were recorded at 14K and at a power level of 5 mW.

Table I: Correlation of Enzyme Activity and Oxidation Level of the MoFe Protein with O_2 Exposure Time[a]

oxidation of MoFe (e/mol)	activity (% of unexposed)	O_2 exposure time (min)
6.0	90–100	3–5
12	65–85	8–12
12	30	20
15–20	~0	>30

[a] $S_2O_4^{2-}$-free MoFe protein was exposed to 10% O_2 in argon for the indicated times after which activity was determined. The oxidation level of the protein was determined by microcoulometry after anaerobic Sephadex G-25 chromatography.

RESULTS

MoFe Protein. The sensitivity of the MoFe protein to various O_2 levels and to fixed O_2 levels monitored as a function of time is shown in panels a and b of Figure 1, respectively. At a given O_2 concentration, the activity of the MoFe protein decreases nonlinearly with time (Figure 1a). It is also seen that, as the O_2 concentration increases under fixed time reaction conditions, there is only a small corresponding decrease in protein activity (Figure 1b). At short exposure times or at longer exposure times but at lower O_2 concentrations, the activity of the MoFe protein is relatively unaffected by O_2. For example, the 5% O_2 curve in Figure 1a indicates that reaction times of up to 5 min cause only a 10% loss in enzyme activity, but as the O_2 concentration increases, this time interval of minimum O_2 inactivation decreases until at 20% O_2 it is less than 2 min.

The addition of 1 mM $Na_2S_2O_4$ to the MoFe protein definitely has a protective effect toward O_2 inactivation (results not shown). Under the conditions of 20% O_2 and 1 mM $S_2O_4^{2-}$, the activity of the $S_2O_4^{2-}$-free MoFe protein shown in Figure 1a at 5-, 10-, and 20 min reaction times is 25%, 24%, and 20% lower than that of the $S_2O_4^{2-}$ containing reaction mixture. After about 20 min, the two types of reactions begin to converge more quickly, and less difference is observed. $S_2O_4^{2-}$ protection clearly occurs at the beginning of the reaction, where presumably O_2 is competing much more effectively for $S_2O_4^{2-}$ than for the MoFe protein, resulting in protection of the latter. When the $S_2O_4^{2-}$ decreases due to reaction with O_2, protein inactivation by O_2 begins to increase until after about 20 min, and the initial protective effect of $S_2O_4^{2-}$ is no longer evident. The addition of 1 mM $MgCl_2$ or a 1:1 ratio of FeS-II to $S_2O_4^{2-}$-free MoFe protein had no significant effect on stabilizing the MoFe protein against O_2 inactivation.

In order to determine where O_2 interacts with the MoFe protein and to evaluate the early events related to O_2 inactivation of this protein, a series of reactions was carried out in which reduced $S_2O_4^{2-}$-free MoFe protein was exposed to 10% O_2 in argon for various time intervals followed by separation of the protein from O_2, O_2 reduction products, etc. by anaerobic G-25 chromatography. The protein was then quantitatively reduced (coulometrically) to determine the extent of oxidation having occurred during O_2 exposure and assayed to relate exposure time with the degree of oxidation and specific activity. Table I summarizes the results of several studies and suggests that at least three partially overlapping reactions occur. During the first reaction, lasting up to 10 min,

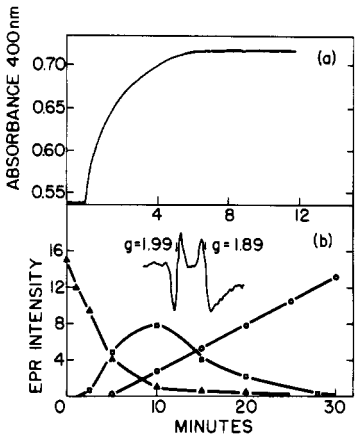

FIGURE 2: O_2 reaction with the MoFe protein monitored spectroscopically. (a) Optical absorbance change at 400 nm of the MoFe protein (5 mg/mL) during the reaction with 10% O_2 in argon. (b) EPR spectral intensity of the MoFe protein (8 mg/mL) at $g = 3.65$ (△), $g = 1.89$ (□), and $g = 2.0$ (○) as a function of time under 10% O_2 in argon. The inset spectrum shows the development of the new EPR signals after 8 min of exposure to 10% O_2 in argon.

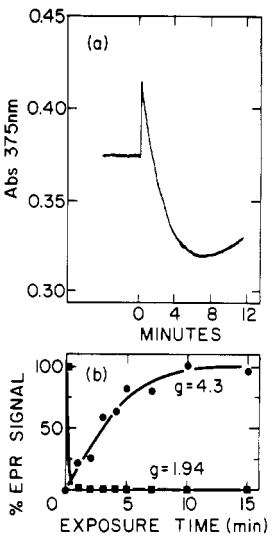

FIGURE 3: O_2 reaction with the Fe protein monitored spectroscopically. (a) Optical absorbance at 375 nm of the Fe protein (6 mg/mL) during the reaction with 5% O_2 in argon. (b) EPR signal intensity of the Fe protein (10 mg/mL) at $g = 1.94$ (■) and $g = 4.3$ (●) as a function of time under 5% O_2 in argon.

as many as 12 electrons are readily removed from the MoFe protein with little or no activity loss. After about 10 min, a second reaction becomes dominant in which no further electron removal occurs but during which activity diminishes at an accelerated rate. The third reaction begins to be important after about 20 min. Activity loss continues to decrease and is accompanied by increased electron loss.

Metal analysis of the MoFe protein first exposed to 10% O_2 in argon until greater than 90% activity loss occurred followed by Sephadex G-25 chromatography showed that no metal loss occurs under these conditions.

Another perspective of O_2 interaction with the MoFe protein is seen in Figure 2. In this figure, the EPR characteristics of the MoFe protein are monitored under 10% O_2 in argon as a function of time (Figure 2b). The typical EPR spectrum of reduced MoFe protein ($g = 4.3$, $g = 3.65$, and $g = 2.01$) decreases with time until after 10 min it is barely detectable. Table I indicates that little activity loss has occurred after 10 min but that protein oxidation up to 12 electrons has occurred. After about 2 min, new EPR signals appear (see insert, Figure 2b), which increases in intensity with time, peak at 10 min, and then decline. A $g = 2.0$ signal begins to form after about 6 min of O_2 exposure and increases, almost linearly, with time for over 60 min. The presence of this latter signal correlates with irreversible activity loss.

A third view of O_2 interaction with the MoFe protein is that in Figure 2a. The absorbance at 400 nm of the MoFe protein increases with time during reaction with O_2. The absorbance stabilizes after about 12 min, indicating that the protein reactions which occur after this time period and which result in activity loss are not detectable by optical spectroscopy at the wavelengths studied. The addition of $S_2O_4^{2-}$ to the protein after a 12-min O_2 exposure decreases the absorbance to near the original value and produces a recovery of 85% of the original activity. The addition of a small amount of methylviologen increases rapidly the reduction by $S_2O_4^{2-}$.

Fe Protein. Parts b and c of Figure 1 show the effect that various O_2 concentrations and exposure times have on the activity of the Fe protein. This protein is clearly much more sensitive toward O_2 than is the MoFe protein. For example, under identical conditions of 10% O_2/90% argon, the time required for 50% loss of the specific activity is 24 min for the MoFe protein and 2 min for the Fe protein. Even if this activity loss is normalized per Fe atom, normalized per cluster present, or adjusted for molecular weight differences, the Fe protein is still the more O_2-sensitive protein. Unlike the MoFe protein, where no measurable activity loss occurs upon initial O_2 exposure, the Fe protein begins to lose activity immediately upon contact with O_2. The Fe protein is also quite different from the MoFe protein in its behavior toward $S_2O_4^{2-}$ under O_2 inactivation conditions. In the presence of 1 mM $S_2O_4^{2-}$, we observed no protective effect for the Fe protein. In most cases, there was an actual increase in inactivation compared to the $S_2O_4^{2-}$-free Fe protein control. This result suggests that O_2 has a much greater affinity for the Fe protein than for $S_2O_4^{2-}$ (present at a 10–50-fold excess) so that the latter affords little protection. Another possibility is that the products of O_2 reduction by $S_2O_4^{2-}$ (O_2^-, HO_2^-, H_2O_2, etc.) also exert a rapid and irreversible inactivation of the Fe protein. We found no difference in the inactivation rate of Fe protein in the presence or absence of 1 mM MgATP under otherwise identical conditions. $MgCl_2$ at 1 mM increased the inactivation of the Fe protein by O_2. FeS-II at a 1:1 ratio with the Fe protein did not exert a protective effect.

Optical and EPR spectral changes of Fe protein exposed to 5% O_2 as a function of time are shown in Figure 3. The spectral changes occur much more rapidly with the Fe protein than with the MoFe protein. The initial rapid rise in the optical spectrum shown in Figure 3a corresponds to an essentially reversibly one-electron oxidation of the Fe protein followed by a much slower decline in absorbance with time which eventually falls below the initial absorbance value. The slow rise after 7 min is due to turbidity of the solution resulting from protein precipitation.

A similar result is seen in Figure 3b where the EPR signal intensity of the Fe protein at $g = 1.94$ and $g = 4.3$ is plotted as a function of time during exposure to 5% O_2 in argon. The signal at $g = 1.94$, typical of active Fe protein, is rapidly lost upon O_2 exposure (<30 s), but Figure 1 shows that negligible loss of activity has occurred. Thus, a simple, nondestructive one-electron oxidation of the Fe protein apparently occurs with full retention of protein activity. This rapid loss of the $g = 1.94$ signal corresponds to the rapid rise in absorbance shown in Figure 3a.

After loss of the $g = 1.94$ signal has occurred (~1 min), a new signal at $g = 4.3$ begins to form. The intensity of this

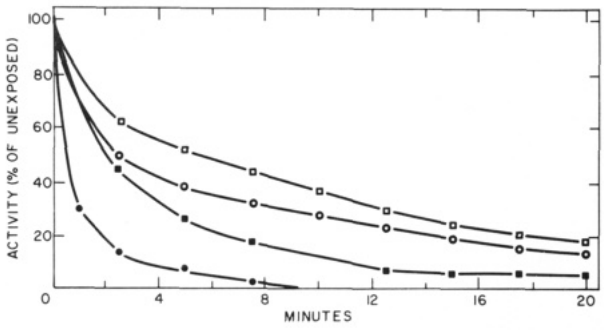

FIGURE 4: O_2 reaction with combined nitrogenase proteins. Fe protein and MoFe protein in 0.05 M Tris–0.25 M NaCl, pH 8, combined in a 1:1 ratio (43 μM in each protein) exposed to 10% O_2 in argon. Activity was monitored in triplicate as a function of time in the absence (■) and presence (●) of 1 mM $MgCl_2$. Same conditions except 1.1 mol of FeS-II was added per mol of MoFe and Fe proteins in the absence (□) and presence (○) of 1 mM $MgCl_2$.

new signal increases with time until, after 10 min, maximum signal development has occurred. Figure 1c shows that protein activity is lost in parallel with the development of the $g = 4.3$ signal in Figure 3b and the slow loss of absorbance seen in Figure 3a. The signal that forms at $g = 4.3$ is typical of high-spin Fe^{3+} in a low symmetry environment.

Metal analysis of Fe protein initially containing 3.65 Fe per mole exposed to O_2 until inactivated and then passed through an anaerobic G-25 column to remove small molecules was found to contain only 2.68 Fe atoms per mole. The $g = 4.3$ signal, which forms upon O_2 exposure, could arise from this easily removed Fe atom. This inactivated, oxidized protein undergoes reduction by $S_2O_4^{2-}$ as evidenced by both optical absorbance decrease and the recovery of the typical EPR spectrum, although neither were at the original intensity.

Fe–MoFe Mixture. Fe protein and MoFe protein were combined anaerobically in a 1:1 ratio and then exposed to various concentrations of O_2 for various times. Figure 4 shows the results obtained at 10% O_2 as a function of time; the other O_2 concentrations that were studied were omitted for clarity of presentation but formed a family of curves similar to that shown in Figure 1. The overall results of O_2 exposure with the 1:1 complex are very similar to those for the Fe protein alone in Figure 1c and suggest that the activity decrease for the 1:1 ratio results from Fe protein inactivation. This was demonstrated by assaying a 1:1 mixture after nearly complete inactivation by O_2 using added active Fe protein. The specific activity of the MoFe protein in this inactive 1:1 mixture after O_2 exposure was same as that prior to O_2 exposure, indicating that no significant loss of catalytic function of the MoFe protein had occurred due to O_2 inactivation. The presence of Mg^{2+} had a detrimental effect in stabilizing the 1:1 ratio against O_2 activation as shown in Figure 4.

Fe–MoFe–FeS-II. The rate of inactivation of a 1:1:1 ratio of Fe protein, MoFe protein, and FeS-II all initially reduced but $S_2O_4^{2-}$ free in 10% O_2 in argon is shown in Figure 4. When the results are compared to those of Fe protein alone (Figure 1c) or to those of a 1:1 ratio of Fe protein and MoFe (Figure 4) under identical conditions, it is seen that the FeS-II protein imparts some stability to the nitrogenase components but that the overall effect is not great. The addition of Mg^{2+} to 1 mM decreases slightly the stability of this three-component protein system toward O_2 inactivation.

Ultracentrifugation. Sedimentation values ($s_{20,w}$) of 4.25 and 10.4 S at 20 °C and zero protein concentration were determined for the Fe protein and MoFe protein, respectively. An average sedimentation value of 12.0 S at zero protein concentration was determined for the Bulen nitrogenase complex from several independent measurements.

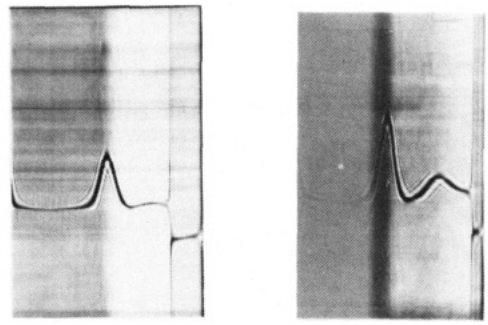

FIGURE 5: Ultracentrifugation patterns of the nitrogenase complex and component proteins. (a) Sedimentation peak of the nitrogenase (Bulen) complex at 8 mg/mL in 0.01 M TES, pH 7.5. (b) Sedimentation peak of the combined components at a 1:1:1 ratio in 0.05 M Tris–0.25 M NaCl, pH 8.0. In both experiments the rotor speed was 48 000 rpm, and the photograph was taken 24 min after rotor speed was attained.

From the extrapolated $s_{20,w}$ value for the nitrogenase complex, a molecular weight of 300 000–335 000 was calculated. Figure 5a is a Schlieren pattern of the nitrogenase complex, and Figure 5b is a similar pattern using a 1:1:1 ratio of the components as found in the Bulen complex [see Lough et al. (1983)]. Figure 5a shows a single symmetrical peak during the sedimentation interval while Figure 5b shows two separately sedimenting peaks. Analysis of these two peaks in Figure 5b gives sedimentation values very near those of the separately run Fe and MoFe proteins. We found no evidence for formation of a higher molecular weight complex from a 1:1 ratio of combined components with or without FeS-II under any of the conditions studied, including 1 mM $MgCl_2$, as long as anaerobic proteins were used.

Exposure of MoFe protein to O_2 resulted in an altered ultracentrifuge pattern consisting of high molecular weight ($>10^6$) rapidly sedimenting protein aggregates of MoFe protein and normally sedimenting MoFe protein. The nature of this aggregation was not determined but was found to be O_2 induced.

A 1:1:1 mixture of MoFe, Fe, and FeS-II proteins separately oxidized with methylene blue and then combined or this same mixture of reduced proteins combined together and then oxidized with methylene blue (in the presence or absence of 1 mM $MgCl_2$) produced peaks in the ultracentrifuge corresponding only to the free Fe and MoFe proteins.

Discussion

The O_2 sensitivity of the nitrogenase complex and its component proteins has been the subject of a number of investigations involving three types of O_2 exposure experiments. Type I studies are few in number (Wong & Burris, 1972) but consist of reacting the nitrogenase complex or its component proteins containing $S_2O_4^{2-}$ with O_2. The complex competition setup between O_2 and the two reactants, $S_2O_4^{2-}$ and the protein(s) under study, makes these experiments difficult to interpret.

Type II and type III experiments both use reduced $S_2O_4^{2-}$-free nitrogenase proteins but differ in the method of O_2 exposure. Type II experiments have no gas phase and require mixing the protein with buffer previously equilibrated under the desired O_2 atmosphere, while type III experiments expose protein directly to an atmosphere containing the desired O_2 partial pressure. Limited O_2 concentration in the type II experiments and faulty gas–liquid equilibration with type III experiments are problems that can produce artifacts and lead

to misleading interpretation of results. For example, when type II experimental protocol is used, it is necessary to demonstrate that the amount of added O_2 exceeds the total reducing equivalents present in the protein mixture. This is particularly important in view of the results reported here that 12 electrons are rapidly and reversibly removed by O_2 from the MoFe protein with little or no loss of enzyme activity. This effect plus smaller contributions from the Fe protein and added FeS-II protein (when used) can decrease significantly the initial O_2 concentration in the reaction mixture before any O_2-inactivation reactions occur. Another caution using this approach is to make certain that the initial added O_2 concentration remains in solution during the reaction period and does not decrease during the establishment of new equilibrium conditions. This can be a severe problem if the solutions are agitated exposed to temperature fluctuation or allowed to stand longer than a few minutes. Coulometric measurements have indicated that when equal volumes of O_2-saturated buffer and O_2-free buffer are carefully mixed in a glass, gas-tight syringe and allowed to remain static, the initial O_2 concentration of this metastable state decreases (more than 40% decrease in 20 min) due apparently to a reestablishment of a new gas–liquid equilibrium condition. A further complication using this procedure is that the rate of reaction between the protein and the O_2 continually changes with time as the latter is consumed by the inactivation reaction. This creates changing kinetic behavior during the reaction interval and complicates the interpretation of the O_2-inactivation results.

For these reasons, we have chosen type III reaction conditions for the studies reported here. For these conditions, we have demonstrated that gas–liquid equilibrium is rapidly established in less than 15 s with no detrimental effects on the proteins and that the total molar amount of O_2 in solution remains essentially invariant and in a near constant excess over the protein concentration during the period of the experiments. Our results represent protein inactivation under zero-order rate conditions with respect to O_2 and therefore should more closely represent the actual O_2 inactivation behavior of the proteins. Analysis of the O_2 inactivation curves at a fixed O_2 concentration indicates that the reactions are approximately first order in protein, suggesting that the O_2 concentration is in fact nearly constant during protein inactivation.

MoFe Protein. Figure 1 shows that this protein is quite stable toward O_2 inactivation especially at short contact times or at low O_2 partial pressures. For example, at 5% O_2 (6.5×10^{-5} M in O_2 in the aqueous phase), where the O_2:MoFe ratio is maintained at $\sim$2, we detected little activity loss after 5 min followed then by only slow inactivation. When $S_2O_4^{2-}$ is present, it has a definite protective effect during the early stages of O_2 exposure probably due to its preferential reaction with O_2. Mg^{2+} or a 1:1 ratio for MoFe and FeS-II has essentially no protective effect on the MoFe protein. O_2 exposure produces aggregation as monitored by ultracentrifugation. Long threadlike masses of polymerized MoFe form under such conditions.

What has generally been lacking in previous O_2 inactivation studies of the MoFe protein are attempts to determine what is the mechanism for O_2 inactivation and what features of the protein are being altered by O_2 exposure. The results of this study provide some insights. Controlled O_2 exposure produces fully active MoFe protein oxidized by 6 (Watt et al., 1981) or 12 electrons (Table I). Both of these oxidation states have been previously described (Watt et al., 1980a,b) by using oxidants more easily controlled than O_2. This result suggests that O_2 behaves as a simple reversible oxidant during the first stages of O_2 exposure by nondestructively oxidizing the metal-containing redox centers in the protein. Spectroscopic measurements confirm this behavior by showing that the original spectroscopic behavior returns after reduction. Subsequent O_2 exposure then produces loss in activity without further oxidation being observed. However, because the degree of oxidation was determined coulometrically, oxidation of groups ($2RSH \rightarrow R\text{–}S\text{–}S\text{–}R$, $S^{2-} \rightarrow S^0$, etc.) not readily reduced by the coulometry conditions would go undetected. Further extensive exposure of the MoFe protein to O_2, during which more than 85% activity loss occurred followed by passage of the protein through Sephadex G-25 columns produced MoFe protein oxidized by more than 20 electrons (as evidenced by well-behaved coulometric reduction), but which had the same metal content as fully active protein. O_2 exposure, therefore, does not destroy the metal clusters as $Fe(CN)_6^{3-}$ does (Gomez-Moreno & Ke, 1979), causing them to be labile with respect to decomposition into their component metal ions. This O_2-inactivated protein still undergoes coulometric reduction at potentials near those of oxidized but active protein, but as reported by Watt et al. (1980a) and confirmed here, the rate of reduction is faster in the inactivated protein.

The EPR signals in the $g = 1.7$–2.0 region which occur in oxidized but fully active MoFe protein (Figure 2) have been noted previously (Palmer et al., 1972; Gomez-Moreno & Ke, 1979) and were found to occur from oxidation with either $Fe(CN)_6^{3-}$ or O_2. We have correlated the development of these signals with the degree of oxidation of the MoFe protein and have found that signal development occurs when oxidation exceeds 6 electrons but is eliminated when oxidation approaches 12 electrons. More extensive oxidation than 12 electrons gives rise to the $g = 2.0$ EPR signal. The rise of this signal during O_2 exposure parallels activity loss and indicates that O_2 irreversibly destroys a protein component that produces this signal. The lack of optical absorbance change with initial activity loss combined with the EPR results suggests that the component undergoing inactivation is nonmetallic in nature, perhaps involving sulfur groups. The observed $g = 2.0$ signal is similar to that reported by Morgan et al. (1984) resulting from $Fe(CN)_6^{3-}$ oxidation of *A. vinelandii* ferredoxin I. These authors attribute the $g = 2.0$ in ferredoxin to the reaction of $Fe(CN)_6^{3-}$ at an iron sulfur cluster forming a cysteinyl disulfide radical (Cys–S–S·). A similar reaction might occur with the iron-sulfur clusters in the MoFe protein.

Fe Protein. The Fe protein is very O_2 sensitive. Even brief O_2 exposure, in the presence or absence of $S_2O_4^{2-}$, causes some irreversible activity loss. Figure 1c shows the details of inactivation with time under the conditions described. Fe protein is inactivated at essentially the same rate in the presence of Mg^{2+}, MgATP, a 1:1 ratio with MoFe, or a 1:1 ratio with FeS-II as it is in the free state. The lack of protection and perhaps an enhancement of inactivation by $S_2O_4^{2-}$ are a curious but unexplained result.

The results with the Fe protein show that it reacts rapidly and, apparently, simply with O_2 initially causing cluster oxidation to occur. O_2 exposure causes some Fe ($\sim$1Fe) to be lost from the Fe protein and produces an absorption spectrum which has features in common with simple 2Fe-2S proteins. This modified Fe protein still undergoes reduction, but more than one electron is required.

Complex Formation from Components. Several studies have investigated the formation of an O_2-stable complex from recombination of the nitrogenase component proteins with FeS-II. Haaker & Veeger (1977) showed that increasing the

ratio of FeS-II in a 1:1 mixture of nitrogenase components increased the O_2 stability under type III conditions. At a ratio of 1:1:2–3, they report a maximum stability at 80% of the unexposed proteins was reached for a 1-min exposure to air. Similar results had been reported earlier (Haaker et al., 1977). What was left unexplained and is quite interesting is why increasing the ratio from 2–3 to about 10 caused complete reversal (Haaker & Veeger, 1977) of the O_2 stabilization effect by this protein on the nitrogenase components.

In a similar study, Robson (1979) showed that about 75% of the original activity remained at a 1:1:1 ratio of nitrogenase components and FeS-II after a 2-min exposure to O_2 under type II conditions. Below a 1:1:1 ratio, O_2 stability decreased rapidly. Although slightly different conditions prevail (especially with Robson's data), these two O_2-exposure studies can be compared with our results for a mixture of the MoFe and Fe proteins with and without FeS-II (see Figure 4).

This comparison reveals that the results of Haaker & Veeger (1977) showing only a 20% decrease in activity with a 3-fold excess of FeS-II over the nitrogenase components after 1-min exposure to air are essentially the same as our results in Figure 4 with only the Fe and MoFe protein. Robson's data for a 2-min exposure to O_2 show a slightly increased stability over our results for the same time of exposure, but because his data were taken under type II conditions and those of Haaker & Veeger (1977) and those in Figures 1 and 4 were type III conditions, we feel Robson's O_2 stability results are overestimated when compared on the same basis with the other results. The O_2 stabilization of the nitrogenase components by the FeS-II protein reported in the two previous studies (Haaker & Veeger, 1977; Robson, 1979) in the presence of a 1–3-fold excess of FeS-II is not very impressive. Our results in Figure 4 at a 1:1 ratio of FeS-II to the nitrogenase components show, likewise, an unimpressive stabilization when compared to the results in Figure 4 obtained in the absence of FeS-II. Only a modest O_2 stabilization is imparted by the FeS-II protein.

The single point, fixed time [1 min (Haaker & Veeger, 1977); 2 min (Robson, 1979)] O_2-exposure experiments may be misleading in their evaluation of the FeS-II O_2-stabilization effect because they are subject to grater experimental error due to the relative short exposure intervals used and because of the steepness of the activity vs. time curves at the early stages of inactivation. We feel that a more reliable assessment of the O_2 stabilization effect on the nitrogenase proteins by the FeS-II protein is made as a function of O_2-exposure time as shown in Figure 4. This approach tends to reduce the experimental errors and also provides a wider view of the phenomenon.

The ultracentrifugation experiments in Figure 5 show no evidence for the formation of a complex with either a 1:1 ratio of Fe protein to MoFe protein or a 1:1:1 ratio of Fe protein, MoFe protein, and FeS-II protein. Under all conditions examined in this study, only separate peaks of the two-component proteins were observed with 1:1:1 mixtures. Sedimentation values of the peaks in the mixtures correspond closely with those for free Fe protein and MoFe protein. No evidence for a component with the 12.0 S sedimentation value observed for the native complex was seen. Taken together, the data presented here do not support the presence of a strongly associated complex formed from a 1:1:1 ratio of nitrogenase component proteins and the FeS-II with or without 1 mM $MgCl_2$ when in the reduced or oxidized state.

This behavior of the combined components contrasts sharply with the behavior observed for the native complex isolated by the Bulen–LeComte (1972) procedure. This entity is composed of a 1:1:1 ratio of the three components with a molecular weight of 330000 (Lough et al., 1983), is much more O_2 stable (Bulen & LeComte, 1972), and is seen in the ultracentrifuge as a single symmetrical peak sedimenting with an $s_{20,w}$ value of 12.0 S (Figure 5), consistent with a molecular weight of 330000. In addition, the reduction of dye-oxidized complex (Lough et al., 1983) gives a two-step redox curve quite different from the redox behavior of the separate nitrogenase components. Reduction of a dye-oxidized 1:1:1 mixture of proteins gives redox behavior approximating reduction of separately oxidized components and not resembling the reduction of the dye-oxidized Bulen complex. These data also support our contention that a strongly associated complex does not form by combination of a 1:1:1 mixture of nitrogenase components and FeS-II to form the equivalent of the native Bulen complex.

The question then becomes, what types of interactions occur and does a complex form other than the Bulen complex by combination of the nitrogenase components and the FeS-II protein to impart that small but measurable O_2 stability observed in Figure 4 and in previously reported studies (Robson, 1979; Haaker & Veeger, 1977)? In a recent paper, Scherings et al. (1983) discuss the formation of a three-component nitrogenase complex that has increased stability toward O_2 inactivation and that is formed by the interaction of the component nitrogenase proteins and the FeS-II protein. This complex has a reported (Scherings et al., 1983) minimum stoichiometry of 1:2:2 MoFe:Fe:FeS-II and an average molecular weight of 1.5×10^6. Simpson & Burris (1983) have reported the formation of a similar redox-dependent, O_2-stable complex of high molecular weight from interaction of the FeS-II protein and the nitrogenase components. Both complexes are more O_2 stable, compared to the components, and are likely to be identical in nature. It is clear then that this high molecular weight complex is quite different from the Bulen complex both in composition and in behavior.

Whether this high molecular weight complex is of physiological significance seems doubtful, but this possibility remains to be further explored. The necessity of preoxidation of the three protein components comprising this nitrogenase complex in order to induce O_2 stability is behavior noted by both Scherings et al. (1983) and Simpson & Burris (1983). The observation by Scherings et al. (1983) that controlled O_2 exposure eliminates the preoxidation requirement is consistent with the results reported here. When both the MoFe and Fe proteins are exposed to low O_2 levels, they undergo reversible cluster oxidation without loss of activity. These results support the hypothesis (Scherings et al., 1980; Simpson & Burris, 1983) that the component proteins must be oxidized to produce the O_2-stable high molecular weight complex.

Registry No. O_2, 7782-44-7; nitrogenase, 9013-04-1.

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Functional and Immunochemical Characterization of a Mutant of *Escherichia coli* Energy Uncoupled for Lactose Transport

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ABSTRACT: Right-side-out cytoplasmic membrane vesicles from *Escherichia coli* ML 308-22, a mutant "uncoupled" for β-galactoside/H^+ symport [Wong, P. T. S., Kashket, E. R., & Wilson, T. H. (1970) *Proc. Natl. Acad. Sci. U.S.A. 65*, 63], are specifically defective in the ability to catalyze accumulation of methyl 1-thio-β-D-galactopyranoside (TMG) in the presence of an H^+ electrochemical gradient (interior negative and alkaline). Furthermore, the rate of carrier-mediated efflux under nonenergized conditions is slow and unaffected by ambient pH from pH 5.5 to 7.5, and TMG-induced H^+ influx is only about 15% of that observed in vesicles containing wild-type *lac* permease (ML 308-225). Alternatively, ML 308-22 vesicles bind *p*-nitrophenyl α-D-galactopyranoside and monoclonal antibody 4B1 to the same extent as ML 308-225 vesicles and catalyze facilitated diffusion and equilibrium exchange as well as ML 308-225 vesicles. When entrance counterflow is studied with external substrate at saturating and subsaturating concentrations, it is apparent that the mutation simulates the effects of deuterium oxide [Viitanen, P., Garcia, M. L., Foster, D. L., Kaczorowski, G. J., & Kaback, H. R. (1983) *Biochemistry 22*, 2531]. That is, the mutation has no effect on the rate or extent of counterflow when external substrate is saturating but stimulates the efficiency of counterflow when external substrate is below the apparent K_m. Moreover, although replacement of protium with deuterium stimulates counterflow in ML 308-225 vesicles when external substrate is subsaturating, the isotope has no effect on the mutant vesicles under the same conditions. It is suggested that the mutation in ML 308-22 results in a *lac* permease molecule with a higher pK_a, thereby either limiting the rate of deprotonation or altering the equilibrium between protonated and deprotonated forms of the carrier. Although antibody 4B1 binds identically with ML 308-225 and ML 308-22 vesicles, monoclonal antibody 4A10R, which is directed against a cytoplasmically disposed epitope in the permease that is partially related to the carboxyl terminus of the molecule, binds to inside-out vesicles from the mutant only 30% as well as it binds to the same preparation from ML 308-225. Since the ultimate carboxyl terminus of the permease does not play a direct role in catalytic activity [Carrasco, N., Herzlinger, D., Mitchell, R., DeChiara, S., Danho, W., Gabriel, T. F., & Kaback, H. R. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4672], it is concluded that the mutation in ML 308-22 probably does not occur in the carboxyl terminus of the permease but causes a conformational change in this region of the molecule.

The *lac* carrier protein (i.e., *lac* permease), the product of the *lac y* gene in *Escherichia coli*, has been solubilized from the cytoplasmic membrane, purified to homogeneity in a completely functional state, and demonstrated to be the only polypeptide required for transport of β-galactosides [cf. Kaback (1983, 1984) and Overath & Wright (1983) for recent reviews]. The permease catalyzes translocation of substrate with H^+ (i.e., symport), and in the presence of a proton electrochemical gradient ($\Delta\bar{\mu}_{H^+}$),[1] it couples the downhill movement

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[1] Abbreviations: $\Delta\bar{\mu}_{H^+}$, proton electrochemical gradient; C, carboxyl; TMG, methyl 1-thio-β-D-galactopyranoside; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; PMS, phenazine methosulfate; *p*-CMBS, *p*-(chloromercuri)benzenesulfonate; diS-C_3-(5), 3,3′-diisopropylthiodicarbocyanine; Dns^6-Gal, 6-(*N*-dansylamino)hexyl 1-thio-β-D-galactopyranoside; NPG, *p*-nitrophenyl α-D-galactopyranoside; RSO, right side out; ISO, inside out; $NaDodSO_4$, sodium dodecyl sulfate; kDa, kilodalton(s).

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