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NITROGENASE

VII. EFFECT OF COMPONENT RATIO, ATP AND H_2 ON THE DISTRIBUTION OF ELECTRONS TO ALTERNATIVE SUBSTRATES

LAWRENCE C. DAVIS, VINOD K. SHAH and WINSTON J. BRILL

Department of Bacteriology and the Center for Studies of Nitrogen Fixation, College of Agricultural and Life Sciences, University of Wisconsin, Madison, Wisc. 53706 (U.S.A.)

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Summary

Some kinetic properties of purified component I (Mo-Fe protein) and component II (Fe protein) of nitrogenase (EC 1.7.99.2) from *Azotobacter vinelandii* have been examined. The apparent K_m values for reducible substrates (0.1 atm for N_2, 0.01 atm for acetylene) and dithionite (0.5 mM) are similar for osmotically shocked cell lysates and purified components. However, the ATP dependence of acetylene and N_2 reduction varies sigmoidally with ATP concentration and as a function of the relative and absolute concentration of components I and II in the assay. Acetylene is reduced in preference to N_2 in competitive assays when component I is in relative excess. Acetylene reduction is not as dependent upon ATP concentration as is N_2 reduction, so that acetylene is also a preferred substrate at lower ATP levels. Hydrogen specifically inhibits N_2 reduction, diverting electrons to acetylene when both substrates are present in the assay.

We propose a model of the enzyme activity, in which the substrates for reduction are bound to component I with electrons being activated by component II. ATP may be involved in activating electrons and in maintaining the appropriate conformation or reduction state of components to allow effective reduction of substrates. The relative rate of reduction of alternative substrates is dependent on the concentration of the particular state(s) capable of reacting with each substrate. The concentration of a particular state of component I is a function of components I, II and ATP.

Introduction

The kinetic properties of nitrogenase (nitrogen: (acceptor)oxidoreductase, EC 1.7.99.2) have been under investigation since Lineweaver et al. [1] showed that there was close adherence to Michaelis-Menton kinetics of N_2 fixation in whole cells of *Azotobacter*. Details of the stoichiometry of the reaction and proposed mechanisms have been the subject of considerable discussion [2—5]. Hardy et al. [6] summarized the information and attempted to present a coherent model of nitrogenase activity. They postulate an active site made up of several distinct parts. One site must be responsible for activation of electrons and is closely coupled to a site for evolution of H_2 in the absence of reducible substrate. There must be another closely-coupled site for reduction of N_2 which partially overlaps the site(s) at which N_3^-, acetylene, CN^-, methyl cyanide and analogs are bound and reduced. An additional site for H_2 binding may overlap the N_2 binding site. Kinetic evidence for this sort of active site model has been presented [7—9].

An alternative to the multisite model is the reaction scheme presented by Bulen and co-workers [2,10]. They suggest that ATP is involved in producing conformation changes of the nitrogenase complex which allow it to react with different substrates. Silverstein and Bulen [2] observed that when low levels of ATP are used in the assay there is more H_2 evolved than N_2 reduced but that when the level of ATP is raised the evolution of H_2 remains constant while the reduction of N_2 increases. This behavior varies as a function of the absolute concentration of the complex and can be explained to be due to a dissociation of the components I and II. Bergersen and Turner [11] have recently presented kinetic evidence consistent with a modification of this reaction scheme for the mechanism of bacteroid nitrogenase activity. Their data are consistent with the assignment of substrate binding sites to component I and an effector function to component II. The apparent K_m for reducible substrates varies greatly with the concentration of component II but relatively little with the component I concentration, implying that component II may modify the ability of component I to bind substrates.

Both components of *Azotobacter vinelandii* nitrogenase have been purified and are highly active [12,13]. Examination of the kinetics of the purified components may allow us to distinguish between the two proposed models. If the model of Bulen [2,10] is correct, the patterns of substrate competition will vary with the relative concentrations of ATP and ratios of components I and II. If the model of Hardy et al. [6] is correct, the substrate competition should be a function only of the concentration of substrates. Changing the concentration of the protein components or of ATP should affect only the ability of the enzyme to activate electrons (V) not the patterns of competition between reducible substrate (K_m and K_i).

We have determined apparent K_m values for several substrates using highly purified components and have examined the mutual competition of acetylene and N_2 in the presence and absence of H_2, which is a specific inhibitor of N_2 reduction [14]. Our results are consistent with a model of the reaction sequence in which ATP and component ratios control the relative ability of the enzyme to reduce different substrates [2].

Methods

A. vinelandii OP was grown and extracts prepared as described previously [15]. Details of procedures for purification of components I and II have been described [13]. Preparations were homogeneous by gel electrophoresis. All chemicals and gases were reagent grade and were obtained from commercial sources.

Assays were carried out as previously described [13]. The assay was linear for at least 30 min and was terminated after an appropriate time (usually 15 min) by addition of 0.1 ml 30% trichloroacetic acid. Ethylene production was measured 30 min later by gas chromatography. Assays for N_2 reduction were stopped with 1 ml saturated K_2CO_3, and ammonia was determined by Nessler reagent after microdiffusion. During simultaneous assays of N_2 and acetylene reduction the reaction was carried out in 22-ml bottles with 1 ml aqueous phase to maximize N_2 reduction. After the appropriate time (15—30 min) the reaction was stopped by adding 0.1 ml 30% trichloroacetic acid and acetylene reduction was determined. 1 ml of saturated K_2CO_3 was then added to the same vials and ammonia produced was determined colorimetrically after microdiffusion.

Dithionite oxidation rates were determined by the method of Ljones and Burris [16]. Protein concentrations were determined by the method of Lowry et al. [17]. For the assays shown in the figures, component ratios are expressed on the basis of activity of each component when it was titrated with an optimum amount of the other component. Specific activities of the purified fractions were greater than 800 nmol/min per mg protein.

Results

Dithionite oxidation

The simplest reductive reaction carried out by nitrogenase is the transfer of electrons from an oxidizable substrate, such as dithionite, to the protons of water with evolution of H_2. Oxidation of dithionite can be conveniently followed by the method of Ljones and Burris [16]. For the relatively higher concentrations of dithionite needed to saturate nitrogenase from *Azotobacter* [9], it is more convenient to follow the oxidation at a longer wavelength where the absorbance is less. An experimentally determined molar absorbance ($M^{-1} \cdot cm^{-1}$) of 1300 at 350 nm was used.

The K_m for dithionite was determined by following dithionite oxidation at 315 nm [16] with varying concentrations of ATP and components I and II of nitrogenase. The apparent K_m for dithionite was about 0.5 mM and was independent of the concentration of ATP or of absolute component concentration at a fixed ratio of components. Plots of the same data for apparent K_m for ATP gave a value of approx. 1 mM. When dithionite oxidation was followed at 350 nm, it was possible to determine rates of acetylene reduction in the same cuvette by repeated intermittent shaking to equilibrate the gas and liquid phase. Rates of acetylene reduction, extrapolated to V, agreed well with the dithionite oxidation rate when an excess of component II was present. At low ATP (below 1 mM) or with limiting component II, the rates of acetylene reduc-

tion were lower than the rates of dithionite oxidation. This is similar to the effect observed by Silverstein and Bulen [2] with N_2 reduction versus H_2 evolution.

Reducible substrates

With the standard assay system (2.5 mM ATP) we found an apparent K_m for acetylene reduction of 0.02 atm, when an excess of component II was present and dithionite oxidation was monitored simultaneously. An apparent K_m of 0.005—0.02 atm was found in the standard assay with enzyme fractions of varying degree of purity. The apparent K_m for N_2 is approx. 0.1 atm. Attempts to determine the K_m for ATP with N_2 as the reducible substrate gave non-hyperbolic plots [2] and the K_m for ATP was estimated to be above 1 mM.

We have previously discussed the effect of component ratios on the relative reduction of acetylene and N_2 under standard assay conditions [13,18]. As the relative amount of component I is increased, the K_m for acetylene is lowered. At very high ratios of component I: component II, acetylene becomes a potent inhibitor and no K_m can be accurately determined. When fixed levels of reducible substrates are used, the evolution of H_2 may not be completely suppressed [7,9] and variations in the extent of substrate reduction may represent either variation in H_2 evolution or inhibition of the overall reaction. We therefore carried out N_2 and acetylene reduction assays with varying concentrations of both substrates in single assay bottles so that the total reduction of substrates, as well as the mutual competition, could be determined.

Mutual competition of reducible substrates and H_2 inhibition

When simultaneous assays of acetylene and N_2 reduction were carried out, it was found that acetylene was preferentially reduced. This preference was greater when component II was limiting. Hwang et al. [8] showed that acetylene is a non-competitive inhibitor of N_2 reduction, while Rivera-Ortiz [19] has shown that N_2 is clearly a competitive inhibitor of acetylene reduction. In both cases, a fixed ratio of components was used (a partially purified extract fraction) and the non-competitive inhibition by acetylene deviated from linearity toward higher acetylene reduction at high levels of acetylene.

Hydrogen has long been known to be a competitive inhibitor of N_2 fixation in *Azotobacter* [20,21]. Hwang et al. [8] showed that H_2 is a specific inhibitor of N_2 reduction only. We therefore compared the relative reduction of acetylene and N_2 in the same assay as a function of the H_2 concentration (Figs 1 and 2). Under the conditions chosen (0.7 atm N_2, varying acetylene) acetylene reduction was competitively inhibited by the N_2 (Fig. 1). Addition of H_2 resulted in a reversal of the inhibition of acetylene reduction, while simultaneously decreasing the reduction of N_2 (Fig. 2). The sum of electrons transferred to reducible substrates was approximately constant (±10%) over the range of H_2 concentrations tested suggesting that H_2 acted mainly to divert electrons from N_2 to acetylene. For these assays we used a fixed ratio of component activities (1.5 parts I:1 part II).

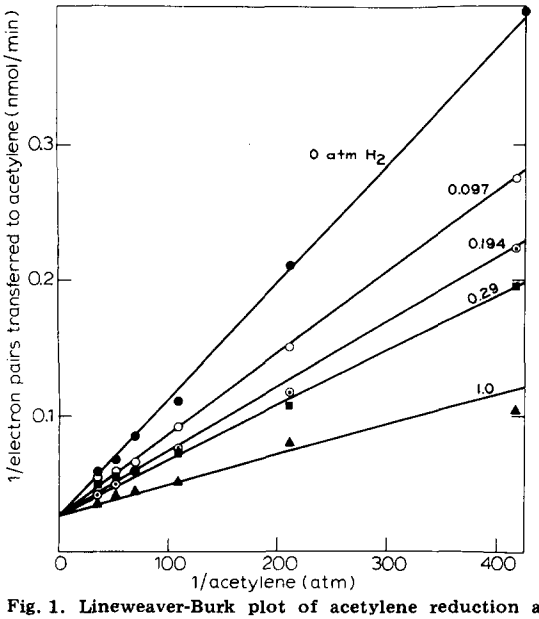

Fig. 1. Lineweaver-Burk plot of acetylene reduction as a function of hydrogen concentration in the presence of nitrogen. Assays were carried out in 22-ml serum bottles containing 0.7 atm N_2, H_2 as indicated and He to make 1 atm. Acetylene was added after removal of an equivalent aliquot of gas phase. Reactions were initiated by addition of enzyme (osmotically shocked lysate) and stopped after 15 min at 30° C by adding trichloroacetic acid. Specific activities of components I and II were 52 and 34 nmol/min per mg protein, respectively. Acetylene reduction is expressed as electron pairs transferred to substrate. Various assay conditions are indicated as follows: ●———●, no addition of H_2; ○———○, 0.097 atm H_2; ⊙———⊙, 0.194 atm H_2; ■———■, 0.29 atm H_2; ▲———▲, 1 atm H_2 or He.

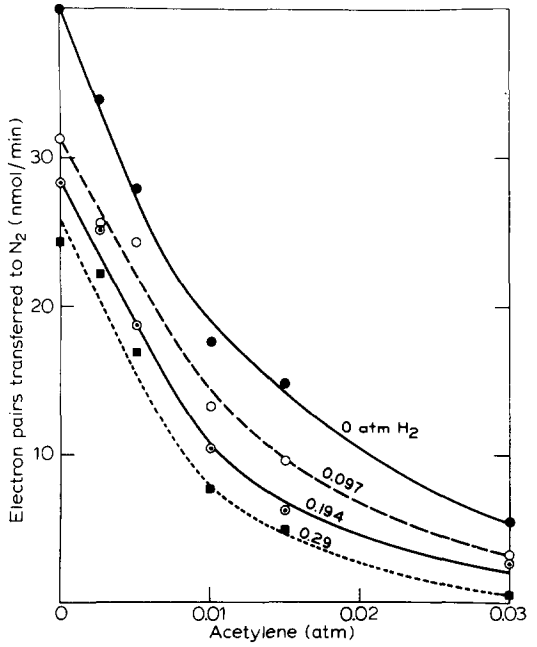

Fig. 2. Nitrogen reduction as a function of hydrogen and acetylene concentration. Assay conditions and enzyme preparations are the same as indicated in Fig. 1. N_2 reduction is expressed as electron pairs transferred to substrate. Various assay conditions are indicated as follows: ●———●, no addition of H_2; ○———○, 0.097 atm H_2; ⊙———⊙, 0.194 atm H_2; ■———■, 0.29 atm H_2.

ATP dependence

Reduction of either acetylene or N_2 requires ATP. However, the ratio of ATP hydrolyzed per pair of electrons transferred to reducible substrates varies as a function of substrate concentration [9] and component ratio [22]. Silverstein and Bulen [2] showed that the saturation curve for ATP is sigmoid during N_2 reduction assays. We found a similar effect on acetylene reduction when component II was limiting. Ljones and Burris [22] showed that the ratio of mol of ATP hydrolyzed per mol of dithionite oxidized increased as the concentration of component I was increased (i.e. component II became limiting). ATP hydrolysis may be inhibited by high levels of acetylene [9]; furthermore, acetylene shows greater substrate inhibition when component II is limiting [18].

Nitrogen and acetylene reduction were compared in a competitive assay system as a function of the ATP concentration (Fig. 3). As expected, N_2 did not affect the V (extrapolated) of acetylene reduction at any ATP concentration tested. In contrast, the relative inhibition of N_2 reduction by acetylene was much more pronounced at low levels of ATP. The sum of electrons transferred to reducible substrates was approximately constant for all acetylene concentrations tested at a given level of ATP, again indicating diversion of electrons from N_2 to acetylene. Thus, the non-competitive nature of acetylene inhibition of N_2 reduction is enhanced by low ATP concentration.

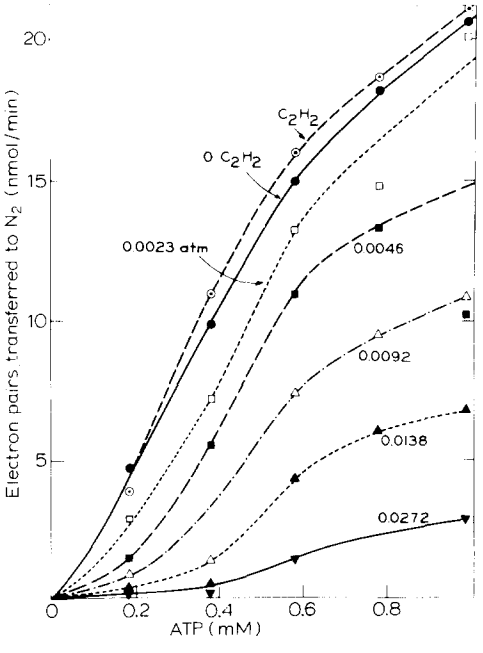

Fig. 3. Nitrogen reduction as a function of acetylene (C_2H_2) and ATP concentrations. Assays were carried out as in Fig. 1 with 0.97 atm N_2 and acetylene as indicated. ⊙———⊙, indicates the average sum of total electrons transferred to acetylene plus N_2, or acetylene alone extrapolated to V. Other symbols show the rate of N_2 reduction (electron pairs transferred) in the presence of the indicated levels of acetylene: ●———●, no acetylene; □———□, 0.0023 atm acetylene; ■———■, 0.0046 atm acetylene; △———△, 0.0092 atm acetylene; ▲———▲, 0.0138 atm acetylene; ▼———▼, 0.0272 atm acetylene.

Effect of component ratios

If, as postulated by Silverstein and Bulen [2], ATP is involved by regulating the association-dissociation and/or conformation of components I and II to produce states of the enzyme which preferentially bind particular substrates, variation of either the component ratios of ATP may affect the distribution of states of the enzyme capable of binding these substrates. We have examined the effect of systematic variation in component ratio, ATP and reducible substrate concentration. For the experiment shown in Fig. 4 the ATP level was held constant at 2.5 mM, N_2 at 0.97 atm and the acetylene concentration was varied for three different ratios of components. Note the difference in the concentration of acetylene required to consume half of the total electron flow to reducible substrates in parts A, B and C. When component II was severely limiting, as in part C, acetylene became a much more effective inhibitor of N_2 reduction, and consumed the bulk of electrons.

To observe the influence of ATP on the pattern of electron utilization, assays were carried out using constant levels of acetylene and N_2, varying ATP and component ratios (Fig. 5). The relative utilization of acetylene compared to N_2 was much greater at low ATP levels although the absolute acetylene utilization did not change greatly over the range of ATP concentrations tested. Comparing Fig. 5 with Fig. 4 for the same ATP and acetylene concentrations, we find quite good agreement for all three component ratios. Since N_2 under these conditions (Fig. 4) cannot completely suppress H_2 evolution, and the levels of acetylene used (Fig. 5) are only about twice K_m, the sum of electrons transferred to reducible substrates did not equal that obtained using saturating

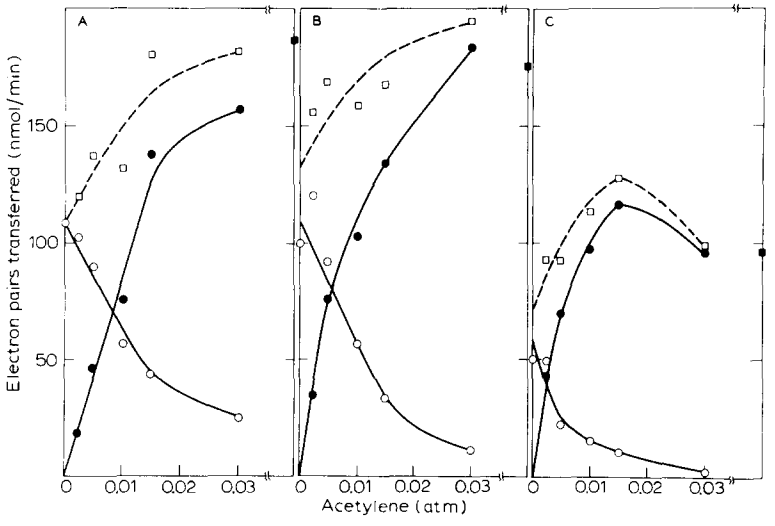

Fig. 4. Variation in nitrogen reduction as a function of acetylene concentration and ratio of components. Assays were carried out as indicated in Fig. 1, using a constant amount of component II per assay. Component II was a Sephadex G-200 fraction with no detectable component I contamination; component I was a crystallized preparation. 200 units (nmol acetylene formed/min per assay) component II was used with 500, 1000, and 2000 units of component I in parts A, B and C, respectively. ●———●, indicates acetylene reduction; ○———○, N_2 reduction; □———□, total electron pairs transferred to acetylene plus N_2; ■———■, acetylene reduction at 0.09 atm acetylene under 1 atm H_2.

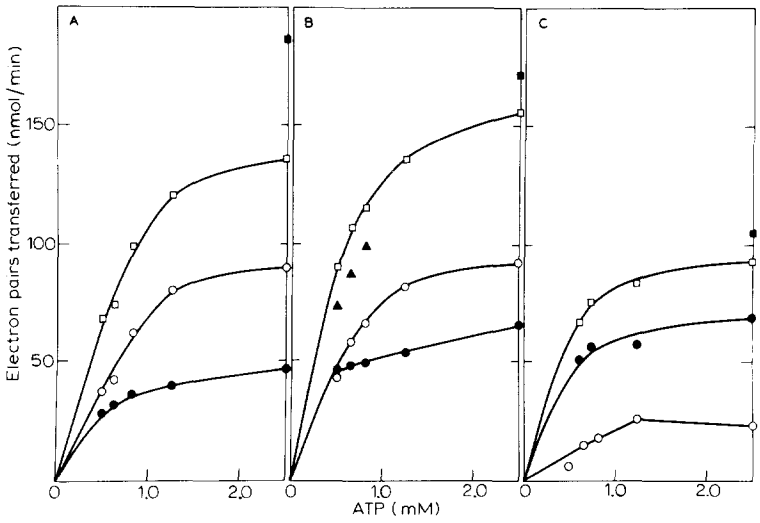

Fig. 5. Electron transfer to substrates with varying ratios of components and varying levels of ATP. Assays carried out as in Fig. 1 with the acetylene concentration constant at 0.0046 atm and ATP levels varied as indicated. The enzyme preparation was the same as in Fig. 4. Data expressed as electron pairs transferred to substrates. ●——●, indicate acetylene reduction; ○——○, N_2 reduction; □——□, total electrons transferred to acetylene plus N_2; ■——■, indicate acetylene reduction at 0.09 atm acetylene under 1 atm H_2; ▲——▲, acetylene reduction at 0.0046 atm acetylene under 1 atm H_2.

acetylene under H_2 (far right of each part of Fig. 5). We cannot determine the efficiency of substrate reduction in this figure.

In Figs 1 and 2 we showed the effect of varying H_2 on the relative reduction of acetylene and N_2 at a fixed component ratio as a function of

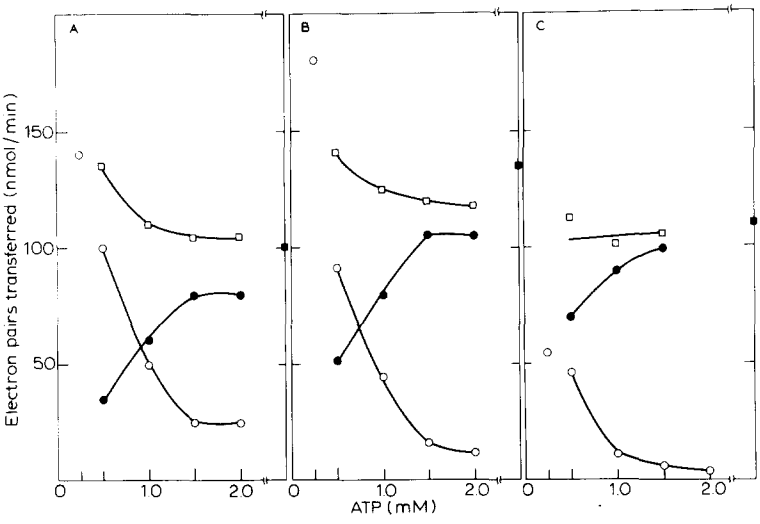

Fig. 6. Effect of varying hydrogen concentration on nitrogen and acetylene reduction with varying ratio of components at fixed concentrations of acetylene and nitrogen. Assays were carried out as indicated in Fig. 1 with 0.70 atm of N_2, 0.0046 atm acetylene, and H_2 as indicated. The enzyme preparation was the same as in Fig. 4. Assay conditions are indicated as follows: ▲——▲, N_2 reduction with no added acetylene or H_2; ○——○ N_2 reduction; ●——●, acetylene reduction; □——□, total electrons transferred to acetylene plus N_2; ■——■, reduction of acetylene at 0.0046 atm acetylene under H_2.

varying acetylene concentration. Fig. 6 shows the results of an experiment using fixed levels of acetylene and N_2, varying H_2 and component ratios. With the component ratios used for this figure, H_2 is a more potent inhibitor of N_2 reduction than under the conditions of Fig. 2 since the ratio of components used makes component II limiting in the assays. Note that the cross-over point, where half of the electrons are diverted to acetylene, occurs at lower H_2 concentrations as the relative limitation of component II is increased.

There are three conditions which appear to have a common effect of diverting electrons from N_2 to acetylene: limitation of component II concentration, limitation of ATP, and the presence of H_2. These three are shown to interact with each other in a cumulative, though not simply additive way.

Discussion

The basic results obtained with purified components of nitrogenase from *A. vinelandii* differ relatively little from those obtained using osmotic lysates of the same organism or fractions of varying degree of purity from other organisms [2–7,21,23]. In confirmation of previous results, we have found that the K_m for acetylene is about 0.01 atm and about 0.1 atm for N_2. Likewise, the apparent K_m of 0.5 mM for dithionite and 1 mM for ATP in the dithionite oxidation assay are in reasonable agreement with the results of others [7,24]. There is a real, reproducible difference between the K_m for dithionite in the *Azotobacter* and clostridial systems [22]. The basis for this difference is presently unknown.

Of more interest to us than apparent K_m values, are the patterns of competition between the substrates, acetylene and N_2. Nitrogen appears to be a simple competitive inhibitor of acetylene reduction [8], while acetylene is a non-competitive inhibitor of N_2 reduction [19]. The deviation from linear non-competitive kinetics is greater at higher concentrations of acetylene, where N_2 at partial pressures approaching 1 atm (10 times K_m) is not significantly reduced in the presence of acetylene at only five times its K_m. Hydrogen emphasizes this property of the enzyme, increasing the relative reduction of acetylene at the expense of N_2. The simplest explanation for this observation is a model with overlapping sites for N_2 and acetylene reduction, with H_2 able to bind to a part of the N_2 site not accessible to acetylene [6,7].

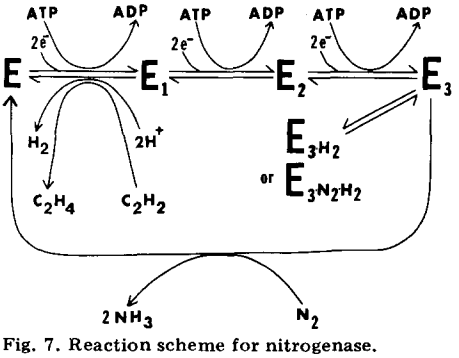

Fig. 7. Reaction scheme for nitrogenase.

An alternative model (Fig. 7) which may help account also for the results obtained with varying ratios of components and levels of ATP is based on that proposed by Silverstein and Bulen [2]. If H_2 binds to a state of the enzyme which is specific for the reduction of N_2 it can be a competitive inhibitor of N_2 reduction only. Such a state could be E_3, or an activated intermediate in the N_2 reduction reaction such as diimide or hydrazide. In this model, the electron transfer mechanism to component I for reduction of alternate substrates may be identical; only the conformation or redox state of the enzyme for binding substrate need change.

In the model of Silverstein and Bulen [2] which proposes that the enzyme passes through several intermediate (e.g. partially reduced) states before binding and reduction of N_2; acetylene, H^+ or other substrates may act to discharge the intermediate to a less reduced state. This results in a shift of the relative distribution of all states of the enzyme. If the complex E must pass through state E_1, which can react with acetylene before it reaches state E_3 which can react with N_2 and H_2, at saturating levels of acetylene the concentration of E_3 may become vanishingly small and acetylene will be a non-competitive inhibitor of N_2 reduction. If, in addition, acetylene can interact with state E_3, acetylene may be "competitive" with N_2 at low concentrations where it does not shift the relative distribution of states appreciably. Substrate inhibition by acetylene at high concentrations [18] further complicates these kinetic studies.

For clarity we have drawn the model omitting many possible reactions such as the reduction of H^+ and acetylene by states E_2 and E_3. It is known that H_2 evolution and ATP hydrolysis vary as a function of component ratio [22] in absence of reducible substrate, which implies that the most reduced form of the enzyme for a given component ratio in the absence of substrate must still be able to discharge its electrons to protons (e.g. E_3 when excess component II is present) at a rate as fast or faster than reduction of substrates. Reduction of acetylene by at least one state less reduced than that reducing N_2 is strongly implied by the non-competitive nature of acetylene inhibition of N_2 reduction and the dependence of the extent of such inhibition by ATP and component ratio. Our model shows only the minimum requirements of our results, and is not meant to exclude binding or reduction of acetylene by the more reduced states.

If we make the assumption that ATP modifies the interaction of component II with component I, both ATP and the relative concentrations of components may determine the concentration distribution of states E_1 to E_3 (Fig. 7). For instance, ATP may be involved in the activation of electrons on component II as an obligatory prior step for binding to component I [24–27], or ATP may be necessary to transfer electrons to component I after binding. The observations that "excess" component II promotes the increased efficiency of substrate reduction but does not increase dithionite oxidation when component I is held constant suggest that substrate binding and the proton accepting sites are on component I.

In Fig. 7, E is a form of component I [26] that can be seen as the g = 4.32, 3.65 and 2.01 signal by electron paramagnetic resonance (EPR) spectroscopy and E_1—E_3 are more reduced states of the functioning enzyme [26]. The number of electrons per component II and mol of component II bound to

component I in any of these states are unknown, as are the number of mol of ATP hydrolyzed or the time of ATP hydrolysis relative to electron transfer from component II to component I to reducible substrate. On the basis of the experiments presented here we cannot prove whether acetylene or H^+ or H_2 may react with one or more than one of the reduction states of the enzyme. When component II is limiting in a reaction, the substrate inhibition by acetylene is greater [18], and the steady state of component I is relatively more oxidized (Orme-Johnson, W.H. and Davis, L.C., unpublished observations). This suggests that acetylene reacts ineffectively with a more oxidized state of component I. Smith et al. [28] have reported that acetylene and protons alter the EPR-visible state of component I, which implies that it may be this state which binds acetylene abortively. The observation of Hwang and Burris [9] that high acetylene inhibits ATP hydrolysis suggests that ATP hydrolysis occurs prior to or simultaneous with acetylene reduction.

Substrates which can use four, rather than six or eight electrons, e.g. methylamine production from cyanide, should be competitive with acetylene and non-competitive with N_2. The distribution of electrons to methylamine versus methane should be a function of component ratios in the assay. Rivera-Ortiz [19] using a fixed ratio of components concluded "the results of the effects of alternative substrates on H_2 evolution, demonstrate that N_2 is unable to completely eliminate H_2 evolution, whereas acetylene and cyanide at infinite concentration are". Our results suggest that the relative suppression by alternative substrates is a function of component ratios so that with an excess of component II, N_2 will completely suppress H_2 evolution.

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