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ELECTRON ALLOCATION TO ALTERNATIVE SUBSTRATES OF *AZOTOBACTER* NITROGENASE IS CONTROLLED BY THE ELECTRON FLUX THROUGH DINITROGENASE *

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Summary

The electron flux through dinitrogenase (MoFe protein, protein containing Mo and Fe) from *Azotobacter vinelandii* controls the relative effectiveness of alternative substrates as electron acceptors in the nitrogenase system. The electron flux through dinitrogenase reductase (Fe protein) or the concentration of MgATP do not directly control electron allocation but rather control it via their influence on the electron flux through dinitrogenase. Kinetic properties of substrate reduction were studied as a function of the electron flux through dinitrogenase. N_2 was most effective at high electron fluxes, whereas H^+ was the most effective acceptor at very low rates of electron flow through dinitrogenase. The K_m for acetylene was dependent on the electron flux through dinitrogenase, whereas the K_m for N_2 was much less sensitive to this electron flux. The lag period before the onset of acetylene reduction was proportional to the turnover time of dinitrogenase, and was approx. 12 times greater than the dinitrogenase turnover time. pH has effects on the electron allocation to substrates beyond that expected from the effect of pH on the electron flux; thus, pH may alter the relative ability of the nitrogenase enzyme system to reduce alternative substrates.

* The nomenclature used in this communication is modified somewhat from that suggested by Hageman, R.V. and Burris, R.H. (1978) (Proc. Natl. Acad. Sci. U.S.A. 75, 2699—2702). The MoFe protein is designated dinitrogenase and the Fe protein dinitrogenase reductase to express the activity of the components of the complete system that is designated nitrogenase. This nomenclature has not been accepted by the International Union of Pure and Applied Chemistry and the International Union of Biochemistry.

Introduction

Nitrogenase catalyzes the reduction of N_2 to NH_3 in an ATP-requiring reaction. The enzyme system also catalyzes ATP-dependent H_2 evolution and the reduction of C_2H_2 to C_2H_4 [1,2]. The nitrogenase system consists of two proteins, the larger containing Mo and Fe (dinitrogenase) and the smaller containing non-heme Fe (dinitrogenase reductase). There seem to be only slight physical differences in the proteins as isolated from different N_2 fixing species [3]. Dinitrogenase from *Azotobacter vinelandii* contains 2 atoms of Mo [4], about 30 atoms of Fe and about 30 acid labile S atoms [5] per $\alpha_2\beta_2$ tetramer of mol. wt. 240 000 [6]. The Fe protein from *A. vinelandii* contains 4 Fe and 4 labile S atoms in an α_2 dimer of mol. wt. 60 000 [7]. The Fe in dinitrogenase reductase is present as an [4Fe-4S] cluster [8], which undergoes a reversible 1 electron oxidation-reduction reaction [9,10]. [4Fe-4S] clusters also have been identified within dinitrogenase [11], and an unusual cofactor present in dinitrogenase contains the Mo, as well as Fe and S [5]. The two Mo in dinitrogenase are well separated [4], and presumably represent separate active sites.

The direction of electron transfer through the nitrogenase system has been shown to be from the source of reducing equivalents ($Na_2S_2O_4$ in vitro) to dinitrogenase reductase, then to dinitrogenase and finally to reducible substrate (H^+, N_2, C_2H_2) [4,12,13]. The electron transfer from dinitrogenase reductase to the dinitrogenase is accompanied by the hydrolysis of ATP [14]. Kinetic evidence was presented in 1973 indicating that dinitrogenase contained the substrate binding sites and dinitrogenase reductase served as an effector [15]. Other work has shown that the flow of electrons through dinitrogenase affects the relative competition for electrons by alternative substrates [16]. While these studies show a role for dinitrogenase in substrate reduction, they cannot eliminate a role for dinitrogenase reductase in the process of substrate reduction.

ATP participates in the electron transfer between the nitrogenase proteins [12,13,17], and ATP hydrolysis accompanies this transfer [14]. However, peculiarities in the kinetics of substrate reduction have been observed that have prompted suggestions for a second role for ATP beyond its role in electron transfer [14,18—21]. Suggested second roles for ATP have included both a catalytic role for ATP in each round of electron transfer [18] and once-only activation of nitrogenase toward the reduction of N_2 or C_2H_2 [21]. No convincing evidence has been offered to date requiring a second role for ATP, but only data that are consistent with, and can be explained by, a second role for ATP. Silverstein and Bulen [22] suggested a model for nitrogenase that would explain the ATP-dependence of the relative ability of nitrogenase to reduce N_2 or H^+. They attributed this ability of ATP to control the relative effectiveness of different substrates to the control by ATP of the proportion of nitrogenase in each of 3 different states. They suggested that ATP controlled the rate at which a reaction occurred; this reaction we now presume to be electron transfer between the nitrogenase proteins. The rate of this electron transfer then would determine in which of the 3 states nitrogenase existed. A high rate of MgATP-dependent electron transfer forced the enzyme predominately into state 3, which could reduce N_2, whereas a slow rate of electron transfer caused

nitrogenase to be in state 2, which did not reduce N_2 but did reduce H^+ to H_2. The enzyme was postulated to be in the resting state, state 1, in the absence of ATP. Other studies [23,24] have supported the idea that there are different states of the nitrogenase enzyme system with different effectiveness in the reduction of alternative substrates. It has been suggested that the relative proportion of nitrogenase in these different states could be controlled by ATP [22], by the ratio of the two nitrogenase proteins to each other [24] or perhaps by other factors.

Bergersen and Turner [15] studied the effect of varying the component ratio on the K_m values for C_2H_2 and N_2. Decreasing the electron flux through dinitrogenase, by decreasing the concentration of dinitrogenase reductase, caused the K_m for either C_2H_2 or N_2 to decrease. Varying the electron flux through dinitrogenase reductase, by varying the dinitrogenase concentration, had little effect on the K_m values. Davis et al. [24] found that at low electron fluxes through dinitrogenase, C_2H_2 was a better inhibitor of N_2 reduction than it was at high electron fluxes; this indicated that the K_i for C_2H_2 decreased faster than the K_m for N_2, although the results of Bergersen and Turner [15] did not indicate this. In agreement with Bergersen and Turner [15], Davis et al. [24] found that C_2H_2 saturated the enzyme system at lower concentrations when the electron flux through dinitrogenase was decreased.

At low electron fluxes through dinitrogenase, there is a prolonged lag phase before the onset of C_2H_2 reduction. These low fluxes have been obtained with low temperature [21], extreme component ratios [21,25,26], heterologous crosses of low activity [20] or by inhibition of an active nitrogenase system with a heterologous protein component [27,28]. These lag phases are much longer than the turnover times of the component proteins. A lag phase for N_2 three times longer than for C_2H_2 has been reported [25], but the existence of such a lag also has been denied [26,28]. During the C_2H_2 lag period, there is a complementary burst of H_2 production such that the total electron flux remains constant [21].

The electron flow through the nitrogenase system has been reported to be independent of the presence of reducible substrates such as N_2 or C_2H_2 [29]. But data have been reported that appear to contradict this, particularly data of Thorneley and Eady [21] indicating a 3-fold increase in total electron flux in the presence of C_2H_2 compared to a pure argon gas phase at 10°C. This effect may be temperature dependent, as the effect was much less at 30°C than at 10°C.

In this paper we will discuss some of the properties of substrate reduction by nitrogenase. In particular, we will consider what factor actually controls the relative effectiveness of alternative substrates in competing for electrons. Substrate saturation curves and the dependence of substrate K_m values upon the electron flux will be examined, as well as the relationship of the lag in C_2H_2 reduction to the electron flux. The studies presented here do not provide final answers in substrate kinetics, but they point out some unifying factors in the patterns of substrate competition.

Materials and Methods

Nitrogenase components from *A. vinelandii* OP were purified by a modification [30] of the method of Shah and Brill [31]. Dinitrogenase had a specific activity of at least 2000 nmol C_2H_4 formed per min per mg and dinitrogenase reductase had a specific activity of at least 1700 nmol C_2H_4 formed per min per mg when measured under our standard assay conditions [16]. Material sources and general methods were as we have described earlier [16]. Azotobacter flavodoxin was purified by a modification of the method of Benemann et al. [32]. Mo was measured by the method of Clark and Axley [33]. The active-site concentration of dinitrogenase reductase was estimated in some cases by the method of Ljones and Burris [34] in which the iron chelator bathophenanthrolinedisulfonate is used to measure the concentration of iron released from dinitrogenase reductase when MgATP is added. Unless otherwise specified, all assays were performed at 30°C, in a 1.0 ml reaction volume in a 22 ml vaccine bottle containing final concentrations of 5 mM ATP, 25 mM creatine phosphate, 0.1 mg/ml creatine kinase, 10 mM magnesium acetate, 50 mM *N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) at a pH of 7.4, and 20 mM $Na_2S_2O_4$. Gas phases were as indicated with argon added to a final pressure of 1 atm. Flavodoxin and nitrogenase components were added as indicated. Experiments were repeated several times to insure that the results were reproducible.

Results

Two major hypotheses exist for the roles of MgATP and the two proteins of nitrogenase [35]. The first of these [29,36] holds that the dinitrogenase reductase-MgATP complex serves only to reduce dinitrogenase in the MgATP-dependent reaction, whereas reduced dinitrogenase is solely responsible for substrate reduction. The second hypothesis holds that in addition to their roles in the reduction of dinitrogenase, MgATP and dinitrogenase-reductase serve specific roles in the reduction of substrates. At least two roles have been proposed for ATP beyond electron transfer between the proteins [18,21]. The nitrogenase system frequently has been treated as forming a long-lifetime catalytically-competent complex [37], and this has carried the implication that both dinitrogenase and dinitrogenase reductase have active roles in the reduction of substrates. If the first hypothesis were true, then the rate of electron flow through dinitrogenase should be the sole determinant of the competition between substrates and inhibitors, whereas if the second hypothesis were true, it would be likely that MgATP and dinitrogenase reductase concentrations should have specific effects on the electron allocation patterns rather than an effect only on the electron transfer rate.

After confirmation that the electron flux through dinitrogenase could, in fact, control the allocation pattern [16], it was necessary to establish whether this electron flux was the sole determinant of electron allocation. A system was required that was reproducible but still sensitive to changes in the allocation patterns. The system chosen consisted of nitrogenase under N_2 plus approximately 0.5% C_2H_2; in this system, H_2, C_2H_4 and NH_3 all are formed. Reactions

were run for 2—60 min to transfer an approximately constant total number of electrons to products. This minimized possible variations in the lag phase before C_2H_2 reduction occurred; the lag phase will be discussed later.

Fig. 1A shows the effect of varying the electron flux by varying the MgATP concentration or by varying the component ratio, whereas Fig. 1B shows the effect of varying the MgATP or reductant concentrations. Results are shown as the fraction of the total number of electrons used for generating each product

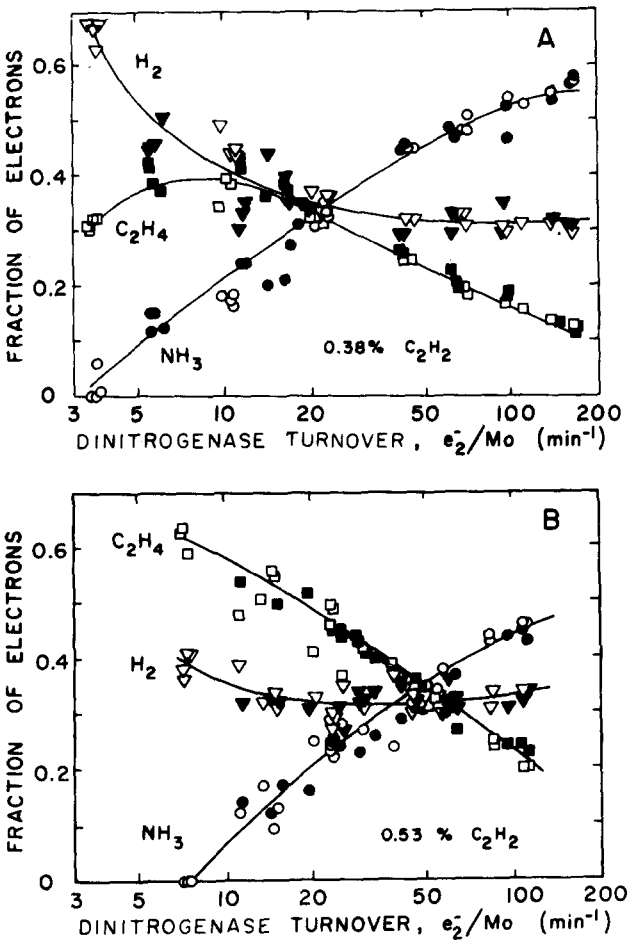


Fig. 1. Electron allocation as a function of the electron flux through dinitrogenase. Standard assay conditions were used with additions as indicated. ○ and ●, e^-_2 in NH_3; △ and ▲, H_2 evolved; and □ and ■, C_2H_4 formed. (A) 100 μM flavodoxin and 5 mM $Na_2S_2O_4$. Open symbols, MgATP varied from 20 μM to 5 mM with 2.55 μM dinitrogenase and 14.2 μM dinitrogenase reductase. The clusters of points from left to right correspond to MgATP concentrations of 20, 30, 40, 70, 100, 200, 500, and 5000 μM. Closed symbols, component ratio varied from 13.4 : 1 to 1 : 5.57 dinitrogenase : dinitrogenase reductase with 5 mM MgATP. The clusters of points from left to right correspond to ratios of components of 13.4 : 1, 5.73 : 1, 2.68 : 1, 1.61 : 1, 1.08 : 1, 1 : 1.86, 1 : 3.72 and 1 : 5.57. The experiment was carried out with 0.38% C_2H_2 in an N_2 gas phase at 30°C. (B) 1.27 μM dinitrogenase and 2.37 μM dinitrogenase reductase. Open symbols, MgATP varied from 40 μM to 5 mM with 5 mM $Na_2S_2O_4$ and 100 μM flavodoxin as reductant. The clusters of points from left to right correspond to MgATP concentrations of 40, 60, 80, 100, 150, 200, 500 and 5000 μM. Closed symbols, reductant varied from 0.6 mM to 5 mM $Na_2S_2O_4$ (plus flavodoxin as indicated) with 5 mM MgATP. The clusters of points from left to right correspond to $Na_2S_2O_4$ concentrations of 0.6, 0.8, 1.0, 1.5, 2.0 (+5 μM flavodoxin), 4.0, 5.0 (+15 μM flavodoxin), and 5.0 mM (+100 μM flavodoxin). The experiment was carried out with 0.53% C_2H_2 in an N_2 gas phase at 30°C.

plotted against the electron flux through dinitrogenase. The total electron flux is obtained by summing the rates of transfer of electron pairs to each substrate and dividing by the concentration of Mo present as dinitrogenase in the reaction mixture. Fig. 1A shows that varying the electron flux through dinitrogenase by varying the MgATP concentration has the same quantitative effect as varying the electron flux through dinitrogenase by varying the component ratio. A plot of electron allocation vs. electron flux through dinitrogenase reductase (plot not shown) does not show such agreement between these two methods of varying the electron flux.

Fig. 1B indicates that varying the MgATP concentration has the same effect on electron allocation as does varying the concentration of reductant. The quantitative differences between Figs. 1A and 1B in the NH_3, C_2H_4 and H_2 curves are due to the different concentrations of acetylene used in the two experiments. A comparison of the three methods for varying electron flux shows that neither dinitrogenase reductase nor MgATP alone have specific effects on the electron allocation to substrates, and these experiments furnish no evidence for a second role for either dinitrogenase reductase or MgATP beyond their roles in electron transfer to dinitrogenase. Under the conditions described for these experiments, the electron flux through dinitrogenase is the sole determinant of the electron allocation pattern. These experiments do not rule out the possibility that pH, temperature or substrate concentration, for example, could have specific effects on the electron allocation pattern.

Fig. 1 also illustrates the qualitative changes in the electron allocation pattern as the flux through dinitrogenase changes. As we [16] and other [24,38] have observed, at very high electron fluxes through dinitrogenase the reduction of N_2 is favored over C_2H_2 reduction or the evolution of H_2. As the electron flux through dinitrogenase decreases, C_2H_2 reduction becomes prominent, and at very low electron fluxes through dinitrogenase the evolution of H_2 is the dominant reaction. Comparison of Figs. 1A and 1B shows that the shapes of the electron allocation curves are dependent on the concentration of acetylene under the reaction conditions used.

It has been reported that the presence of substrates such as N_2 or C_2H_2 does not alter the total rate of electron transfer from $Na_2S_2O_4$ to products [29]. However, indications that this might not be true in all circumstances have surfaced, particularly in the work of Thorneley and Eady [21]; they found a 3-fold increase of total electron transfer at 10°C with C_2H_2 as a substrate relative to H_2 evolution under argon. The effect was much less pronounced at 30°C, and no effect was observed with N_2 as a substrate. Thus, reinvestigation of the effects of substrates on total electron flux was desirable.

Fig. 2A records a C_2H_2-saturation curve, and it indicates that both H_2 and C_2H_2 are formed under the conditions employed. The total electron transfer rate goes through a minimum at 0.05 atm C_2H_2, and then increases. CO inhibits reduction of substrates other than H^+ by nitrogenase [39], and it completely blocks the inhibiting and enhancing effect of C_2H_2 on total electron transfer. There was a very high electron flux through dinitrogenase under the conditions depicted in Fig. 2A; at lower electron flux the inhibition of electron transfer by C_2H_2 is less pronounced but still is noticeable (data not shown). Note that at 0.10 atm C_2H_2 the total electron flux is essentially the same as under argon.

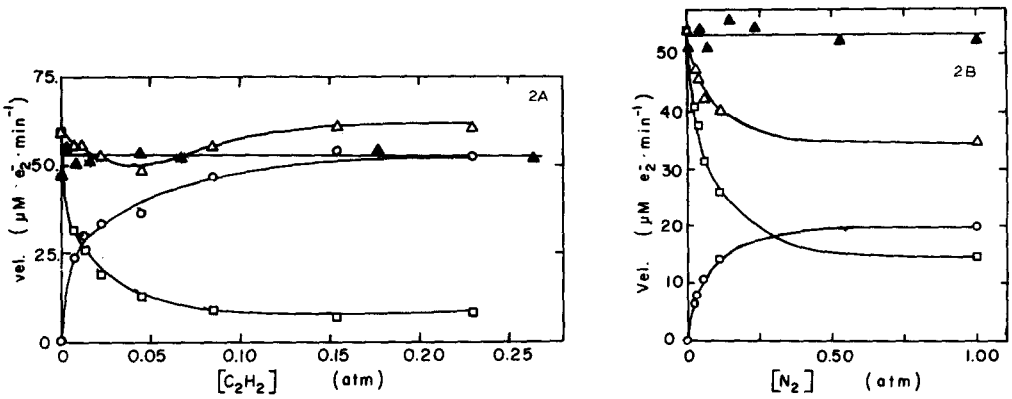


Fig. 2. Substrate saturation curves. Open symbols, uninhibited; closed symbols, inhibited by 1% CO. (A) C_2H_2 saturation curve with standard assay conditions with 5 mM MgATP and 10 mM $Na_2S_2O_4$, 0.160 μM dinitrogenase and 3.94 μM dinitrogenase reductase. ○———○, C_2H_2 reduced; □———□, H_2 evolved; and △———△, total electron flux. (B) N_2 saturation curve under conditions described for Fig. 1A. ○———○, e^- in NH_3; □———□, H_2 evolved; and △———△, total electron flux.

This is a commonly used concentration of C_2H_2 and may account for the failure of other investigators to see the inhibition by C_2H_2.

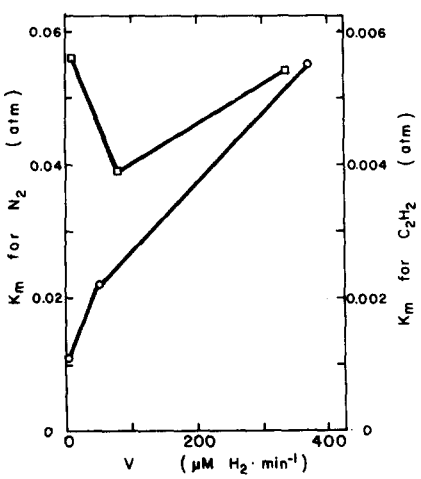
The effects observed with N_2 as a substrate are similar (Fig. 2B) to the initial portion of the C_2H_2 saturation curve. At the high electron flux shown, a pronounced inhibition of total electron flux occurs up to 1.0 atm N_2. CO again blocks this inhibition of total electron flux. Unlike C_2H_2, N_2 at high levels does not enhance electron flux, and this may reflect a fundamental difference in the mechanisms of reduction. However, sufficiently high N_2 concentrations may not have been tested. The highest N_2 concentration was about 16 times the K_m for N_2, whereas the highest C_2H_2 concentration was 60—300 times the K_m for C_2H_2.

Bergersen and Turner [15] have thoroughly studied the dependency of the K_m values for N_2 and C_2H_2 on the concentration of the component proteins. However, they used an impure preparation from *Rhizobium japonicum*, so it is difficult to relate their qualitative observations with quantitative measurements of electron flux. Davis et al. [24] studied the variation of the C_2H_2 saturation curve with change in component ratio and found that the C_2H_2 curve indicated saturation at lower concentrations when dinitrogenase was in excess. Knowing that the determining factor for electron allocation is the electron flux through dinitrogenase, we reinvestigated the dependence of the K_m values for substrates on the electron flux.

Fig. 3 shows the dependency of the observed K_m for substrate reduction on the total electron flux through dinitrogenase. Total electron flux (x axis, Fig. 3) was defined by H_2 evolution under argon. If the total electron flux had been defined as the flux at saturating substrate concentration, the shape of the curves would have been virtually identical to those shown, but they would have been shifted horizontally somewhat to the left of the curves shown. The use of H_2 evolution under argon to define the electron flux allows the comparison of the N_2 and C_2H_2 effects. Use of electron flux at a specific substrate concentration could give confusing results because of possible inhibition or enhancement

of total electron flux by substrates (see Fig. 2A). For C_2H_2 only the data below 0.05 atm of C_2H_2 were utilized to eliminate the uncertain interpretation of the enhancement at high C_2H_2 concentrations. The observed changes in the K_m for C_2H_2 with changes in electron flux is in general agreement with the results of Bergersen and Turner [15]. However, no definite trend with changing electron flux in the K_m for N_2 was observed, which is in contrast to the results reported by Bergersen and Turner [15]. The increased effectiveness of C_2H_2 as an inhibitor of N_2 reduction at low electron flux through dinitrogenase observed by Davis et al. [24] is consistent with little or no change in the K_m for N_2 when the K_m for C_2H_2 decreases. The reason for the discrepancy between the results reported here and the results of Bergersen and Turner [15] is unknown.

An initial lag in C_2H_2 reduction has been reported by other workers [20,21, 25—27], with an accompanying burst in H_2 evolution to maintain a constant total electron flux [21]. A similar lag for N_2 has been reported [25], but it also has been denied [26,28]. The lag in C_2H_2 reduction is much longer than the turnover times of the two nitrogenase proteins. Data presented by Thorneley and Eady [21] indicated that the lag period might be proportional to the turnover time of dinitrogenase, but not dinitrogenase reductase [21]. Before the onset of C_2H_2 reduction we have observed a distinct lag phase, with a complementary burst of H_2 evolution (data not shown). There also is a lag before N_2 reduction begins (Fig. 4). The lag before N_2 reduction is harder to demonstrate than the lag before C_2H_2 reduction; a high rate of NH_3 formation is easy to measure but gives a short lag period, whereas a low rate of NH_3 formation is difficult to measure but gives a longer lag. The conditions described in Fig. 4



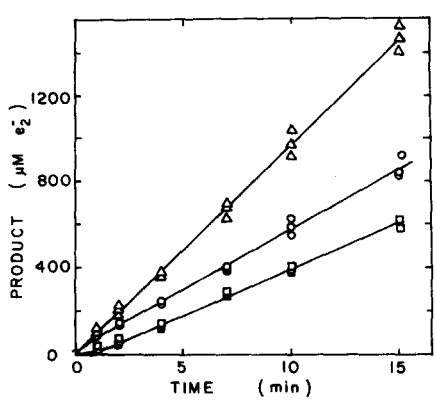


Fig. 3. Dependency of the K_m values for C_2H_2 and N_2 on the total electron flux through dinitrogenase as described in the text. ○———○, K_m (C_2H_2) (right axis); □———□, K_m (N_2) (left axis); K_m expressed in atm.

Fig. 4. Lag period before N_2 reduction. Standard assay conditions were used with 20 mM $Na_2S_2O_4$ as reductant in a N_2 gas phase, 4.53 μM dinitrogenase (6.57 μM molybdenum) and 0.945 μM dinitrogenase reductase (0.50 μM Fe_4S_4) in each assay. Assays were stopped at indicated time by the addition of HCl and analyzed for H_2 and NH_3 formed. □———□, e^-_2 in NH_3; ○———○, H_2 evolved; and △———△, total e^-_2 in products.

seem nearly optimal for detection of the lag in our system. There was a complementary burst of H_2 evolution during the lag in N_2 reduction such that the total electron flux remained constant.

Table I shows the effect of the electron flux through dinitrogenase on the length of the lag in C_2H_2 reduction. The ratio of the lag period to the turnover time shows no obvious trend and averages about 12. The data support a slow activation of dinitrogenase for C_2H_2 reduction; the activation occurs at a rate proportional to the rate of total electron transfer. Table I also shows that the N_2 and C_2H_2 lag periods are approximately of the same length at the same electron flux.

The effect of pH on the electron allocation pattern was investigated to determine whether pH had an influence similar to component ratio and reductant or ATP concentrations. Fig. 5 shows how pH affected the electron allocation and the total electron flux. Above pH 7 the trend in allocation with a change in total electron flux is the same as for the other variables reported in Fig. 1, but below pH 7 changes in the allocation pattern are different from those observed above pH 7. In particular, more C_2H_2 is reduced with a corresponding decrease in N_2 reduction than at the same electron flux above pH 7. That the differences below pH 7 vs. above pH 7 are real is supported by the observation of a change in the lag in C_2H_2 reduction (Table II). Above pH 7 the ratio of the lag period to the dinitrogenase turnover time approaches 10—12, as indicated earlier, whereas below pH 7 the ratio is as low as 3.8. Thus, pH can have a significant effect on the rate of the activation of dinitrogenase for substrate reduction, at least with C_2H_2 as substrate. A third response of nitrogenase to pH is apparent in the curves for N_2 saturation that yield a ratio of H_2 evolved to N_2 reduced as a function of N_2 concentration. Fig. 6 shows the effect of varying the pH on the reciprocal plot of the ratio. At the two higher pH values the ratio extrapolates to a value of 1 at infinite N_2, whereas at the lower pH there is considerably more H_2 evolved than N_2 reduced at infinite N_2. At the highest pH shown, the slope of the line (reciprocal plot) is considerably greater than at the other pH values, indicating that at high pH the $N_2 : 2H^+$ ratio requires a higher concentration of N_2 for saturation. The pH plays an important role in determining the reactions of dinitrogenase with substrates. Note that the

TABLE I

EFFECT OF ELECTRON FLUX ON THE C_2H_2 LAG

Turnover time calculated by [molybdenum] $\times v^{-1}$ where [molybdenum] is the concentration of molybdenum in the assay as dinitrogenase, and is expressed on an e^-_2 per Mo basis. Electron flux was varied by altering the component ratio.

	v (μM e^-_2/min)	Dinitrogenase (μM)	Lag (min)	Dinitrogenase Turnover (min)
C_2H_2 reduction	6.00	9.59	22.6	2.32
	7.15	4.79	14.5	0.97
	14.3	4.79	4.1	0.49
	48.2	4.70	2.3	0.141
	93.9	4.60	0.7	0.071
N_2 reduction	97.6	4.53	0.8	0.067

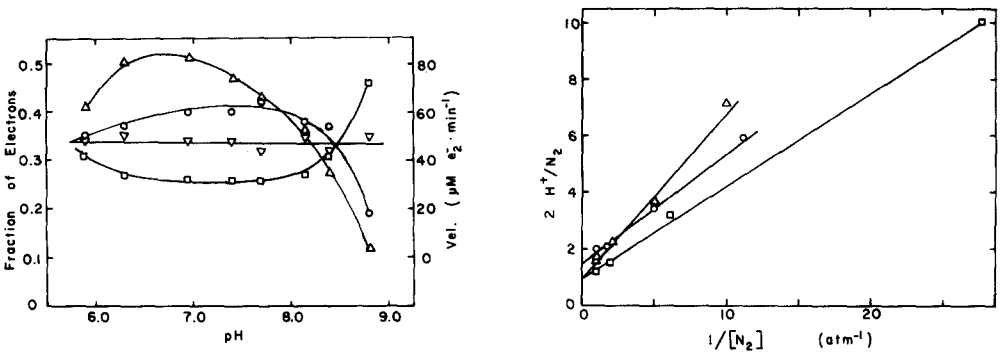


Fig. 5. Effect of pH on electron allocation. Standard assay conditions were used with 20 mM $Na_2S_2O_4$ as reductant, 0.77 μM dinitrogenase, 0.95 μM dinitrogenase reductase and 0.43% C_2H_2 in N_2 gas phase. Allocations were calculated as for Fig. 1. ○———○, e^-_2 in NH_3; □———□, C_2H_4 formed; △———△, H_2 evolved all on left axis; △———△, total electron flux on right axis. All buffer concentrations were 50 mM. pH 5.90, Mes buffer; pH 6.30, Mes buffer; pH 6.95, Mops buffer; pH 7.40, Hepes buffer; pH 7.70, tricine buffer; pH 8.15, bicine buffer; pH 8.40 serine buffer; and pH 8.80 serine plus glycine buffer. Mes, 4-morpholineethanesulfonic acid; Mops, 4-morpholinepropanesulfonic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

Fig. 6. Effect of pH on the N_2 : $2H^+$ ratio. Standard assay conditions were used with 20 mM $Na_2S_2O_4$ as reductant, and a high ratio of dinitrogenase reductase to dinitrogenase present to maximize the allocation into NH_3. ○———○, pH 6.95 with 4-morpholinepropanesulfonic acid (Mops) buffer; □———□, pH 7.70 with tricine buffer; △———△, pH 8.15 with bicine buffer.

experiments in Fig. 6 were designed to maximize the amount of N_2 reduced, but there is no indication that it is possible to reduce more than one N_2 for each H_2 evolved.

Discussion

The early work by Silverstein and Bulen [22], and later work by Davis et al. [24], showed that the kinetic parameters of the enzyme system varied as a function of ATP concentration or the ratio of the component proteins. Hageman and Burris [16] showed that the kinetic effects could be controlled by the electron flux through dinitrogenase. Fig. 1 presents evidence that the electron flux through dinitrogenase is the sole determinant of the electron alloca-

TABLE II

EFFECT OF pH ON THE C_2H_2 LAG PERIOD

pH 5.90, 50 mM Mes; pH 6.95 , 50 mM Mops, pH 7.70, 50 mM tricine; pH 8.40, 50 mM serine. Dinitrogenase turnover defined as in Table I; all assay mixtures contained 4.60 μM dinitrogenase and 0.95 μM dinitrogenase reductase with 20 mM $Na_2S_2O_4$ as reductant.

pH	Dinitrogenase Turnover (min)	Lag (min)	Lag/Turnover
5.90	0.092	0.35	3.8
6.95	0.073	0.57	7.8
7.70	0.073	0.84	11.5
8.40	0.110	1.07	9.7

tion pattern to alternative substrates, and thus this flux controls the kinetic properties of nitrogenase. Concentrations of MgATP and reductant and the component ratio are important only in that they affect the electron flux through dinitrogenase. This assumes importance because it means that any second role for dinitrogenase reductase or MgATP, for example an activation for substrate reduction [20,21,25,26], must occur at a rate proportional to the rate of electron transfer through dinitrogenase, and requires that dinitrogenase reductase, MgATP and reductant be present simultaneously. As any such second role will be kinetically indistinguishable from electron transfer, evidence based on kinetic variations [21,25,26] cannot be used to support it. Although evidence from the cross reactions of heterologous components may be valid [20], it does not specifically support a second role for MgATP or dinitrogenase reductase, as all the effects observed with the heterologous crosses also have been observed with homologous crosses of nitrogenase components.

Above pH 7 the effects of pH variation are the same as the effects of MgATP variation (Fig. 5), namely the favoring of H_2 evolution at very low electron flux and the favoring of N_2 reduction at highest electron flux. Below pH 7 deviations in this pattern are apparent, and a disproportionate amount of C_2H_2 is reduced compared to the same electron flux above pH 7. The differences below pH 7 are borne out by the responses to pH variations reported in Table II and Fig. 6.

Electron allocation was studied at a constant substrate concentration, and the results do not define the effects of substrate concentration on the total electron flux or upon electron allocation to substrates. However, the noncompetitive inhibition of N_2 reduction by C_2H_2 does indicate that variations in substrate concentrations can affect the electron allocation pattern. Thorneley and Eady [21] indicate that reducible substrates other than protons will affect the total electron flux. Fig. 2 shows that either N_2 or C_2H_2 reduces the total electron flux through the *A. vinelandii* nitrogenase system. This inhibition is related to substrate reduction, because 1% CO, which inhibits reduction of substrates other than H^+, counteracts the inhibitory effect of N_2 or C_2H_2. Decreasing the electron flux through dinitrogenase decreases the inhibition. Total electron flux may be inhibited by substrates because the reduction of these substrates becomes rate limiting at high electron fluxes or because substrates induce a conformational change in the nitrogenase proteins to a form that only supports a slower rate of some independent rate limiting process. The increase of total electron flux at high C_2H_2 concentrations may result from activation of a second binding site for C_2H_2. A second site for C_2H_2 has been reported in EPR experiments in which separate high and low affinity sites were found [40].

The results of Bergersen and Turner [15] on the dependency of the K_m for C_2H_2 are supported by our observations, however their results on N_2 are not. This discrepancy may reflect differences in the two systems used or experimental error. If the discrepancy should be resolved as a real difference in the behavior of N_2 and C_2H_2 it could have important mechanistic implications. The observed C_2H_2 saturation kinetics are as predicted for a ping-pong type of mechanism [41]. In such a mechanism, dinitrogenase would be reduced by the dinitrogenase reductase-MgATP complex; the nitrogenase complex then would

dissociate, and the reduced dinitrogenase would reduce C_2H_2. The responses observed in studying the N_2 saturation kinetics may result from a requirement that N_2 or intermediates in N_2 reduction [42] be present simultaneously with the reduced dinitrogenase reductase-MgATP. The kinetics are compatible with a sequential type of reaction.

Any mechanism must account for the long lag period before substrate reduction starts. The lag appears to be correlated with changes that occur in the EPR signal of dinitrogenase within the same time [40]. Activation could occur by slow transfer of electrons from the electron transfer pathway for H_2 evolution into a site that allows binding of C_2H_2 (or N_2) only when the site is in the reduced state. Once reduced, this center would bind substrate and block further H_2 evolution. This once-only reduction could be followed by slow reoxidation of the site after the electron supply from dinitrogenase reductase was cut off. H_2 evolution must be blocked in any scheme to explain how substrates inhibit total electron transfer. The source of electrons for reduction of substrates probably is identical to the source for H_2 evolution. CO might function as an inhibitor either by stopping the reduction of the binding site or by blocking the binding of substrates to this center while still allowing the evolution of H_2. If CO stops reduction of the binding site, then inhibition by CO should start slowly when CO is added after the C_2H_2-lag period.

The dependence of the lag in C_2H_2 reduction on pH suggests that the different pH-dependent forms of dinitrogenase [40] could generate specific substrate binding forms as observed in EPR studies [40], at substantially different rates. The pH dependency of the $N_2 : 2H^+$ reduction ratio may aid in describing the mechanism of N_2 reduction.

Our observations emphasize the importance of the total electron flux through dinitrogenase in controlling the substrate reduction kinetics of the nitrogenase system, and in addition they indicate specific pH effects. The K_m for C_2H_2 appears to vary as expected if one accepts that dinitrogenase itself reduces substrates after its reduction by dinitrogenase reductase. The lag phase before C_2H_2 reduction could arise from an internal electron transfer to a substrate binding site; this internal conversion is an alternative to the external activation proposed by others [20,21,25,26]. The controlling nature of the electron flux through dinitrogenase on the electron allocation pattern precludes the idea that steady state kinetic results can be invoked to support the external activation hypothesis. Further studies on the dependency of substrate kinetics on total electron flux may clarify other aspects of the kinetics of the nitrogenase reaction.

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