Kinetic Studies of Nitrogenase from Soya-Bean Root-Nodule Bacteroids

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The apparent Michaelis constants [$K'(\text{N}_2)$ and $K'(\text{C}_2\text{H}_2)$] and the corresponding apparent maximum velocity values (V') for soya-bean bacteroid nitrogenase increased concomitantly in response to increases in nitrogenase Fe-protein concentration and ATP concentration in cell-free assays and in response to O_2 pressure in intact nodules and bacteroid suspensions. $K'(\text{C}_2\text{H}_2)$ in cell-free assays was also affected by pH and by $\text{Na}_2\text{S}_2\text{O}_4$ concentration. Nitrogenase Fe-protein behaved as a catalytic effector reacting at interacting sites on the nitrogenase Fe-Mo-protein. The results indicated that the Fe-Mo-protein probably bears the catalytic sites for N_2 and C_2H_2 reduction. It is concluded that reduction of N_2 or C_2H_2 by this nitrogenase involves a reaction mechanism with a sequence of unknown order. The sequence in which substrate, enzyme, effector, ATP and reductant react determines which of the various rate-constants are involved in the apparent Michaelis constant, whose true kinetic meaning was thus unresolved.

Nitrogenase from soya-bean bacteroids, like nitrogenases from other micro-organisms, consists of two protein components and catalyses the reduction of H^+ to H_2 , N_2 to NH_3 , C_2H_2 to C_2H_4 and other ATP- and reductant-dependent reactions. One of these components has a molecular weight of about 180000 and contains Fe and Mo. The other, with a molecular weight of about 50000, contains Fe (Koch *et al.*, 1967; Klucas *et al.*, 1968; Bergersen & Turner, 1970). Neither of these proteins has any apparent activity alone and it has been difficult to assign any separate role to either of them. There are suggestions that the N_2 is bound and the electron density between the atoms diminished, in association with the Fe-Mo-protein of nitrogenase and that the Fe-protein is concerned in the reduction of the N_2 activated in this way (e.g., Bui & Mortenson, 1968; Matkhanov *et al.*, 1969; Leigh, 1971; Chatt & Richards, 1971). Kelly & Lang (1970), however, found that both of the proteins, and ATP and reductant, were required for cyanide binding by *Klebsiella pneumoniae* nitrogenase, but Evans *et al.* (1971) concluded that only ATP was required for cyanide binding by the Fe-Mo-protein of *Chloropseudomonas ethylicum* nitrogenase. The role of ATP in nitrogenase-catalysed reactions is obscure also. In addition to its role as a substrate, Kennedy (1970) proposed a conformational role for ATP in lupin bacteroid nitrogenase, related to that originally proposed by Bulen *et al.* (1965). There also appear to be thermodynamic reasons for the involvement of ATP in energetics of reductant supply (Bergersen, 1971).

Studies of the apparent Michaelis constant [$K'(\text{N}_2)$] for intact nitrogen-fixing organisms have yielded values differing by almost an order of magnitude, and values for nitrogenase in cell-free systems are usually higher still (Burris, 1971). Similar variability in the $K'(\text{C}_2\text{H}_2)$ is noted by Hardy (1972).

In work with intact nodules (Bergersen, 1962) and bacteroid suspensions (Bergersen & Turner, 1968), concomitant variation of $K'(\text{N}_2)$ and the apparent maximum velocity (V') has been reported under the influence of variations in O_2 concentration. No evidence has been found for reversal of product formation in legume nodule systems (Bergersen, 1969; Kennedy, 1970) and no free intermediates between N_2 and NH_3 occur (Burris, 1971).

In the work described in the present paper, we have attempted to resolve some of the properties of soya-bean root-nodule nitrogenase by studying the effects of varying the concentrations of some of the components involved in nitrogenase assays.

Experimental

Production of bacteroids

Nodules were produced on soya-beans (*Glycine max* Merr. cultivar Lincoln) as described previously (Bergersen & Turner, 1970) and homogenized anaerobically under argon in a Sorvall Omni-Mixer in which the 400ml chamber was modified by the provision of stainless-steel tubes for argon flushing

and a large-bore, gas-tight valve fitted to the bottom to allow the homogenate to be passed anaerobically through a filter. The filter consisted of a modified sterilizing filter, of diameter 11.5 cm, fitted with two layers of nylon bolting cloth (200 mesh) and made gas-tight with neoprene O-rings and gaskets. After 15 min of flushing with argon, while submerged in the homogenizing medium (125 ml; Koch *et al.*, 1967), the nodules (200 g) were homogenized and, after being left for 5 min, the homogenate was passed through the filter under slight argon pressure. The bacteroids were sedimented from the filtrate by centrifugation at 5000g in argon-flushed capped-tubes and resuspended and washed once in 25 mM-Tris-HCl buffer, pH 7.4.

Nitrogenase preparation

The preparation of bacteroid cell-free extracts and the separation of the Fe-Mo-protein and the Fe-protein components of nitrogenase by anaerobic gel chromatography were done as described previously (Bergersen & Turner, 1970). Partially purified unfractionated nitrogenase was prepared from crude, unheated bacteroid extracts by a method similar to that described by Dalton *et al.* (1971). To 20 ml of bacteroid extract containing 320–400 mg of protein, 1.7 ml of a degassed dispersion of protamine sulphate (50 mg/ml in 25 mM-Tris-HCl buffer, pH 8.0; Mann Research Laboratories, New York, N.Y., U.S.A.) was added dropwise with agitation under a stream of argon. The precipitated acidic proteins and nucleic acids were removed by centrifugation and then a further 1.35 ml of protamine sulphate was added. The dark brown-black precipitate obtained by centri-

fugation was then resuspended anaerobically in 7 ml of buffer and treated with 3 ml of a degassed suspension of washed phosphocellulose (Whatman P 11, 150 mg/ml in 25 mM-Tris-HCl buffer, pH 8.0, adjusted to this pH with NaOH). After centrifuging, the nitrogenase, with a specific activity of 40–60 nmol of NH_3 /min per mg of protein, was located in the supernatant.

Nitrogenase activation

Loss of activity of the nitrogenase Fe-protein during storage of extracts at about 0°C or at room temperature (20°C), and during purification, has been a consistent problem with nitrogenase prepared from strain CB1809 bacteroids in this laboratory. Treatment of crude or partially purified nitrogenase or of Fe-protein preparations, with dithiothreitol (Calbiochem, Los Angeles, Calif., U.S.A.) plus Fe^{2+} , as $(\text{NH}_4)_2\text{SO}_4 \cdot \text{FeSO}_4 \cdot 6\text{H}_2\text{O}$ (BDH Chemicals Ltd., Poole, Dorset, U.K.), resulted in increased activity (cf. Dua & Burris, 1965). This restoration was effective even with preparations in which no activity could be detected before treatment (Table 1), but the treatment usually increased the activity of active preparations by about 50–100% (e.g. Table 2). The addition of $\text{Na}_2\text{S}_2\text{O}_4$ sometimes effected further slight improvement. Restoration of activity was usually completed in 1 h. In the work described below, treatment with dithiothreitol, Fe^{2+} and $\text{Na}_2\text{S}_2\text{O}_4$, each at a final concentration of 5 mM, was applied as a routine to all nitrogenase Fe-protein preparations and to unfractionated nitrogenase, 60–90 min before use, to ensure maximum activity.

Table 1. *Effects of activation reagents on the Fe-protein of soya-bean bacteroid nitrogenase*

Assays, as described in the text, contained 1.3 mg of Fe-Mo-protein, which had been stored in liquid N_2 . The Fe-protein had also been stored in liquid N_2 and thawed slowly under flowing argon; it was treated as shown for 60 min before assaying; the activation reagent concentrations were 5 mM. The concentration of Fe-protein used in these assays gave close to maximum rates of C_2H_2 reduction when treated, and therefore the specific activities shown are relatively low (see Klucas *et al.*, 1968).

Treatment	Activity (nmol of C_2H_4 /min per mg of Fe-protein)
Immediately after thawing	0
60 min after thawing	0
After treatment with:	
$\text{Na}_2\text{S}_2\text{O}_4$	0
Dithiothreitol	0
Fe^{2+}	1.4
$\text{Fe}^{2+} + \text{Na}_2\text{S}_2\text{O}_4$	4.2
$\text{Fe}^{2+} + \text{Dithiothreitol}$	25.6
$\text{Fe}^{2+} + \text{Dithiothreitol} + \text{Na}_2\text{S}_2\text{O}_4$	26.3
Dithiothreitol + $\text{Na}_2\text{S}_2\text{O}_4$	0

Assays

Reduction of N_2 and evolution of H_2 were measured in small flasks fitted with side arms, as described previously (Turner & Bergersen, 1969). Reduction of C_2H_2 was measured in glass vials, each of known volume (about 35 ml) and stoppered with Suba-Seals. In addition to nitrogenase, reaction mixtures contained $MgSO_4$ (6 μ mol or equimolar amounts with ATP), ATP (sodium salt, 6 μ mol or amounts indicated; Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.), creatine phosphate (80 μ mol), creatine kinase (4 units; Sigma Chemical Co., St. Louis, Mo., U.S.A.) and $Na_2S_2O_4$ (25 μ mol), in 2.0 ml of Tris-HCl buffer (25 mM, pH 7.4). The final pH in these assays was 7.1–7.2. The activity of the creatine kinase ATP-generating system represented at least a 10-fold excess over the maximum rate of ATP utilization in these experiments and thus the initial ATP concentration was maintained throughout experiments in which ATP concentration was examined. The reaction vessels were incubated at 25°C with shaking and reactions were terminated by the injection of 0.3 ml of trichloroacetic acid (10%, w/v) after 10 or 15 min, during which reaction rates were constant. The gas phase was argon to which the required amounts of $^{15}N_2$ or C_2H_2 were added by injection to give a final pressure of 93 kN/m². In all the kinetic experiments, substrate concentrations were selected which ranged closely about the value of K' . Similarly, CO concentrations when used ranged closely about the K_i value. Only under these conditions were reproducible results obtained and several preliminary experiments were often required to establish the correct conditions.

Analytical methods

Nitrogen fixation and H_2 evolution were measured mass spectrometrically and acetylene reduction was

assayed by gas chromatography, as previously described (Turner & Bergersen, 1969; Bergersen, 1970; Hardy *et al.*, 1968). Protein was measured by the Folin-phenol reagent (Lowry *et al.*, 1951) with bovine serum albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K.) as standard. Substrate and inhibitor concentrations are given as μ M values calculated from partial pressures and solubilities of the gases in water at 25°C (Washburn, 1928), to minimize the apparent effects of the wide differences in solubility of the gases used. The values used (101 kN/m², 25°C) were 0.64 mm for N_2 , 41.9 mm for C_2H_2 and 0.96 mm for CO.

Statistics

Values of K' , K_i and V' were obtained from Lineweaver-Burk plots (Dixon, 1953) in which the intercepts and the standard errors of the derived values were determined by the method of Wilkinson (1961). Calculations of regression coefficients, axis intercepts, standard errors of slopes of regression lines and probability coefficients were made by standard methods.

Results

Kinetic effects of activation

Since the Fe-protein was to be treated to restore activity as a routine, it was necessary to establish that the reagents to be used would not affect the kinetics of nitrogenase-mediated reactions. This was done to our satisfaction by several experiments such as that illustrated in Table 2. In this experiment, a preparation of the Fe-protein having limited activity in the assay was divided and treated with activation reagents at zero and three increasing concentrations

Table 2. *Effects of activation reagent concentration on K' and V'*

Assays (2.0 ml) contained 1.45 mg of Fe-Mo-protein and 2.0 mg of Fe-protein (0.5 ml). Activation was obtained by treatment of samples of Fe-protein with the indicated equimolar concentrations of dithiothreitol, Fe^{2+} and $Na_2S_2O_4$ for 1 h at 23°C. For other assay details, see the text. Results are means $\pm$ s.d. for four measurements. Significance of differences between reagent concentrations was measured by Student's *t* test; n.s., not significant.

Reagent concentration (mM)	K' (μ M- C_2H_2)	V' (nmol of C_2H_2 /min per assay)
0	4.5 $\pm$ 2.9 ($P < 0.01$)	14.5 $\pm$ 1.6 ($P < 0.01$)
1.25	31.2 $\pm$ 13.0 (n.s.)	41.1 $\pm$ 9.3 (n.s.)
2.5	27.2 $\pm$ 2.3 (n.s.)	41.8 $\pm$ 1.9 (n.s.)
7.5	23.7 $\pm$ 1.1	37.6 $\pm$ 0.8

Fig. 1. Effects of various Fe-Mo-protein concentrations

(a) and (b), Plots of $1/v$ versus $1/[\text{N}_2]$ for N_2 fixation in two similar experiments using 2.8 and 2.6 mg of Fe-protein per assay respectively and the amounts (mg) of Fe-Mo-protein indicated on each plot. (c) and (d), Similar plots for C_2H_2 reduction in two experiments using 2.8 mg of Fe-protein per assay. For other assay details, see the text. (e) Hill plots for N_2 from the data of (a). (f) Hill plots for C_2H_2 from the data of (c).

for 1 h. Portions (2.0 mg of protein) from each were then assayed for activity by using 1.5 mg of Fe-Mo-protein. Apart from the initial effect on K' and V' owing to the increase in the amount of effective Fe-protein present in the assay, the reagents were without further kinetic effects. The reasons for the initial effect will become apparent in the subsequent paragraphs. Treatment of Fe-Mo-protein in the same way was without effect on activity in the assays

and did not affect the kinetic quantities being measured.

Kinetic effects of varying the concentrations of nitrogenase components

Experiments were conducted in which K' and V' values were estimated in assays in which the amount of one of the nitrogenase proteins was kept constant

Fig. 2. Effects of various Fe-protein concentrations

(a) and (b), Plots of $1/v$ versus $1/[N_2]$ for N_2 fixation in two similar experiments using 1.7 and 2.4 mg of Fe-Mo-protein per assay respectively and the amounts (mg) of Fe-protein indicated on each plot. (c) and (d), Similar plots for C_2H_2 reduction in two experiments using 1.7 and 1.9 mg of Fe-Mo-protein per assay respectively. For other assay details, see the text. (e) Hill plots for N_2 from the data of (a). (f) Hill plots for C_2H_2 from the data of (c).

and that of the other varied, using non-limiting concentrations of ATP and $Na_2S_2O_4$. Under these conditions, varying the concentration of the protein containing the enzyme catalytic site(s) should not affect K' , although V' would obviously be affected. However, varying the concentration of an essential component affecting the catalytic step may change K' . Representative plots are given in Figs. 1 and 2. These show that the effects of increasing the Fe-Mo-protein concentration are quite different from the

effects of increasing the Fe-protein concentration. The double-reciprocal plots showed that K' increased with increasing Fe-protein concentration (Fig. 2), but not with increasing Fe-Mo-protein concentration (Fig. 1). Hill plots with N_2 and C_2H_2 as substrates had slopes of close to unity at all concentrations of Fe-protein. Fig. 3 shows the correlations between K' and V' that were obtained from similar experiments. The vertical intercepts obtained are in agreement with results plotted from some other

Fig. 3. Effects of various concentrations of nitrogenase components on K' and V'

(a) N_2 fixation; varied Fe-protein concentration, constant Fe-Mo-protein. $r = 0.97$, slope 0.94 ± 0.16 , $P < 0.01$, $K'_0 = 8.7 \mu M$. (b) C_2H_2 reduction; varied Fe-protein concentration, constant Fe-Mo-protein. $r = 0.94$, slope 0.85 ± 0.28 , $P < 0.05$, $K'_0 = 10.9 \mu M$. (c) N_2 fixation; varied Fe-Mo-protein concentration, constant Fe-protein. $r = 0.67$, slope 0.25 ± 0.16 , n.s. ($P = 0.2$). (d) C_2H_2 reduction; varied Fe-Mo-protein concentration, constant Fe-protein. $r = 0.38$, slope -0.19 ± 0.13 , n.s. ($P > 0.1$). In all cases the regressions were calculated on the basis of two experiments (■, ●) similar to those illustrated in Fig. 1 and Fig. 2. In (a) and (c), values obtained from a third experiment (○) using different component concentrations are also shown. Vertical and horizontal lines indicate the magnitudes of $\pm$ s.d. for each K' and V' value. For experimental details, see the text.

individual experiments in which concentrations of nitrogenase components were different from those in Fig. 3. The results of Fig. 3 have been statistically evaluated and the positive slopes of the lines in Figs. 3(a) and 3(b) are significant ($P < 0.01$ and $P < 0.05$ respectively) whereas those of Figs. 3(c) and 3(d) do not differ significantly from zero ($P > 0.1$).

Plots of V' against Fe-protein concentration were invariably sigmoidal (Fig. 4). The results were evaluated by double-reciprocal plots of V' and Fe-protein concentration for N_2 and C_2H_2 reduction Figs. 4(c) and 4(d). They were concave upwards and were used to estimate $V'_{Fe-prot.}$, the velocity for saturating Fe-protein, and saturating substrate. This value was used in Hill plots (Levitski & Koshland,

1969), where the slopes of all lines were found to give values of n of about 2 [Figs. 4(e) and 4(f)], indicating that the Fe-protein may react at interacting sites on the Fe-Mo-protein.

In other experiments it was found that varying the nitrogenase component concentrations to give two different K' and V' values did not produce any change in the apparent inhibitor constant (K_i) for CO in reduction of either N_2 or C_2H_2 (Table 3).

Kinetic effects of varying the ATP concentration

In contrast to the saturation curves of the reducible substrates of nitrogenase, ATP saturation curves for lupin nitrogenase have been reported to have a

Fig. 4. Effects of Fe-protein concentration on V' (N_2) and V' (C_2H_2)

(a) and (b), Sigmoidal response of N_2 fixation and C_2H_2 reduction to increasing Fe-protein concentration, substrates non-limiting. (c) and (d), Plots of $1/V'$ (N_2) and $1/V'$ (C_2H_2) versus $1/[Fe-protein]$. (e) and (f) Hill plots of Fe-protein. Results for (a), (c) and (e) are from three N_2 -fixation experiments (■, ○, ●) using 0.3 mg of Fe-Mo-protein. Results for (b), (d) and (f) came from a single C_2H_2 -reduction experiment. In all experiments V' was determined from Lineweaver-Burk plots of $1/v$ versus $1/[N_2]$ or $1/[C_2H_2]$. For experimental details, see the text.

marked inflexion (Kennedy, 1970). Similar results were obtained in our work with soya-bean nitrogenase when we used unfractionated protamine sulphate preparations for both N_2 fixation and C_2H_2 reduction. Plots of $1/V'$ versus $1/[ATP]$ were concave upwards and Hill plots of ATP were inflected (Fig. 5). Values of K' increased as V' increased under the influence of increasing ATP concentration. K'

and V' values were again positively correlated (Fig. 6) and the vertical intercepts (K'_0) were similar to those obtained when the Fe-protein concentration was varied (Fig. 3). Hill plots were prepared to see if there was any effect of ATP concentration on the catalytic sites for N_2 or C_2H_2 reduction. The ATP concentration did not affect the slopes of the plots, all of which were close to unity.

Table 3. K_i (CO) measured at different concentrations of nitrogenase components

Results are from two separate experiments from plots of $1/v$ against [CO] at two substrate concentrations (Dixon, 1953). K' and V' are uninhibited values.

Substrate	Nitrogenase		K' (μM)	V' (nmol/min)	K_i (μM)
	Fe-Mo-protein (mg/assay)	Fe-protein (mg/assay)			
N_2	0.50	1.82	22.1	37.0	0.11
	1.51	3.64	32.4	48.8	0.12*
C_2H_2	0.45	1.48	77.1	93.5	0.28
	1.34	2.96	137.8	370.4	0.34*

* The differences between these pairs of K_i values were not significant ($P > 0.1$).

Effects of $p\text{O}_2$ in intact systems

The vertical intercepts in plots of apparent K' against apparent V' obtained from the above experiments were fairly consistent (Figs. 3 and 6); also varying the $p\text{O}_2$ in intact systems could be expected to affect ATP concentrations in the bacteroids in intact nodules or in suspensions. Therefore $K'(\text{N}_2)$ and V' values of intact nodules between 20–51 kN/m² of O_2 (Bergersen, 1962) and of bacteroid suspensions between 2.8–11.2 kN/m² of O_2 (Bergersen & Turner, 1968) were plotted (Fig. 7). Again, the vertical intercepts (K'_0) in these plots were consistent with values obtained with bacteroid cell-free systems (Figs. 3 and 6).

Kinetic effects of varying the $\text{Na}_2\text{S}_2\text{O}_4$ concentration

In the work described in the present paper, $\text{Na}_2\text{S}_2\text{O}_4$ was used as reductant in all cell-free assays. Although this artificial electron-donor may not have the same kinetic effects as natural electron-donors in the nodule bacteroid system, the effects of $\text{Na}_2\text{S}_2\text{O}_4$ concentration were briefly examined by using unfractionated nitrogenase preparations and C_2H_2 reduction. Concentrations of $\text{Na}_2\text{S}_2\text{O}_4$ from 1.25 to 6.24 mM did not affect double-reciprocal plots (Fig. 8). However, 12.4 mM- $\text{Na}_2\text{S}_2\text{O}_4$ was inhibitory, tending to increase K' , and 25 mM- $\text{Na}_2\text{S}_2\text{O}_4$ was markedly inhibitory.

Effects of pH

$K'(\text{C}_2\text{H}_2)$ and V' were measured at pH 6.12, 6.97 and 7.52, by using unfractionated nitrogenase. Effects on V' were negligible but $K'(\text{C}_2\text{H}_2)$ was lower at pH 6.97 than it was at pH 6.12 or 7.52 (Fig. 9). It was not possible to determine whether this effect was due to effects on the enzyme or to altered con-

centration of H^+ affecting the reduction of the substrate, or to a combination of effects.

Discussion

General

Studies of the biochemistry or physiology of nitrogen fixation have included measurements of the apparent Michaelis constant (K') since the work of Lineweaver *et al.* (1934) and Wilson (1936). These and other papers assumed that the K' provided an indication of the affinity of the enzyme for N_2 and the values were used in the comparison of different N_2 -fixing systems. It is now known that nitrogenases from all sources so far studied are remarkably similar, being complex enzymes catalysing several reactions and requiring ATP, bivalent cations and low-potential reductants. The work described in the present paper shows that K' is not constant, but is dependent on the concentrations of nitrogenase Fe-protein, ATP, $\text{Na}_2\text{S}_2\text{O}_4$ and the pH in cell-free assays and, as was shown previously (Bergersen, 1962; Bergersen & Turner, 1968), K' depends on the $p\text{O}_2$ in intact nodule systems or bacteroid suspensions. These observations indicate that differences between $K'(\text{N}_2)$ or $K'(\text{C}_2\text{H}_2)$ values measured in cell-free systems and in natural systems (Burris, 1971) may result from the failure of the artificial systems to match natural concentrations of the components of the reactions. In the experiments described above, 12.5 mM- $\text{Na}_2\text{S}_2\text{O}_4$ was used in most assays. This concentration appeared to be optimum for initial rates of C_2H_2 reduction in preliminary experiments, but the kinetic experiments showed it to be inhibitory (Fig. 9), and thus increasing the K' value. In other work on soya-bean bacteroid nitrogenase (Evans & Russell, 1971), 20 mM- $\text{Na}_2\text{S}_2\text{O}_4$ was used. It is possible that one of the ionic species (such as HSO_3^-) present in $\text{Na}_2\text{S}_2\text{O}_4$ solutions may be an inhibitor (Lyons &

Fig. 5. Effects of ATP concentration, substrate non-limiting

(a) and (b), Response of N_2 fixation and C_2H_2 reduction to ATP concentration. (c) and (d), Plots of $1/V'$ (N_2) and $1/V'$ (C_2H_2) versus $1/[\text{ATP}]$. (e) Hill plots of ATP for N_2 fixation. (f) Hill plots of ATP for C_2H_2 reduction. Results for (a), (c) and (e) are from an experiment using 0.25 mg of a protamine sulphate nitrogenase preparation per assay in which $^{15}\text{N}_2$ fixation was measured. In (b), (d) and (f) C_2H_2 reduction by 1.65 mg of nitrogenase per assay was measured. V' in both experiments was determined from Lineweaver-Burk plots of $1/[\text{N}_2]$ or $1/[\text{C}_2\text{H}_2]$ versus $1/v$. For other experimental details, see the text.

Nickless, 1968). Use of this reductant may thus contribute to high K' values in cell-free assays.

Effects of ATP

Our results are in substantial agreement with those of Kennedy (1970), suggesting that ATP

modifies nitrogenase reactions. However, the results are different from those of Silverstein & Bulen (1970), whose Hill plots from ATP and H_2 evolution had slopes of close to 2 in the concentration range 0.025–0.125 mM. It is possible that this difference may reflect fundamental differences between the *Azotobacter vinelandii* and legume root-nodule bacteroid nitrogenases.

Fig. 6. Plots of K' (N_2) and K' (C_2H_2) versus V' obtained by using up to 4.5 mM-ATP

The ATP concentrations (mM) used to establish each point are indicated in parentheses. Other experimental details are as in Fig. 5.

Fig. 7. Plots of K' versus V' for intact systems with various O_2 pressures

(a) From results of Bergersen (1962) with intact nodules and pO_2 values of (from left to right) 140, 210, 280 and 350 mmHg (19–47 kN/m²). (b) From results of Bergersen & Turner (1968) with bacteroid suspensions and pO_2 values of (from left to right) 21, 42, 53 and 84 mmHg (2.8–11.2 kN/m²).

Location of the catalytic site(s) for N_2 and C_2H_2

There are three possibilities to consider.

(i) The sites may be located on the Fe-protein of this nitrogenase. Our results show that both K' and V' increased with increasing Fe-protein concentration as a fixed amount of the Fe-Mo-protein became progressively saturated (Fig. 2). Expressions for V' contain a function of enzyme concentration, but those for K' do not, therefore increasing the Fe-protein concentration would not increase K' if this protein bore the catalytic site(s).

(ii) The sites may be generated only in a complex

of the two nitrogenase proteins. In this case also, increasing the Fe-protein concentration in the presence of a fixed amount of Fe-Mo-protein would result in an increasing concentration of catalytic sites and should not increase K' unless substrate binding was also affected. Although there was positive interaction between Fe-protein sites on the Fe-Mo-protein (Fig. 4), there was no indication of an increase in numbers of substrate sites per molecule of Fe-Mo-protein from the slopes of Hill plots with N_2 and C_2H_2 as substrates, with increasing Fe-protein concentration [Figs. 2(e) and 2(f)]. We

Fig. 8. Effects of $\text{Na}_2\text{S}_2\text{O}_4$ concentration on K' (C_2H_2)

Assays contained 4mg of unfractionated nitrogenase and the following reductant concentrations: ■, 1.25mM; ○, 3.75mM; ●, 6.25mM; △, 12.5mM; ▲, 25mM. Other assay details are described in the text.

Fig. 9. Effects of pH on K' (C_2H_2)

Assays contained 0.5mg of unfractionated nitrogenase. All reactants in the assay were adjusted to the appropriate pH values. Values of pH shown (■, 6.12; ●, 6.97; ○, 7.52) were measured by glass electrode before reactions commenced but after all reactants were mixed.

conclude, therefore, that it is unlikely that the substrate sites are present only in a complex of the two proteins.

(iii) The remaining possibility, that the Fe-Mo-protein bears the substrate sites, is consistent with the failure of its concentration to affect K' [Figs. 1, 3(a) and 3(b)]. None of the results is inconsistent with the conclusion that the Fe-Mo-protein should be regarded as the enzyme and the Fe-protein as a catalytic effector reacting at two or more positively interacting sites on the Fe-Mo-protein.

These interpretations depend on the effects described being due to the Fe-protein itself and not to unidentified modifier(s) in the preparations, which were not of very high purity. The effects were opposite to those expected from non-specific protein concentration affecting the chemical activity of the substrate as considered by Laurent (1971). Further, the specific effect of the activation agents (Table 2), whose kinetic effect in activating the preparation was the same as adding more Fe-protein, argues against the kinetic effect being due to other undetected

Scheme 1. A reaction sequence for H_2 evolution by nitrogenase

This sequence is derived from the work of Silverstein & Bulen (1970) from studies of *Azotobacter vinelandii* nitrogenase.

Scheme 2. Possible reaction sequences for N_2 or C_2H_2 reduction or by soya-bean bacteroid nitrogenase

In these sequences Fe-Mo-protein is regarded as the enzyme (E), the Fe-protein is considered to be a catalytic effector (modifier, M), reacting at only two interacting sites; N_2 (or C_2H_2) is the substrate and ATP and reductant are non-limiting.

Fig. 10. Equilibrium diagrams and rate equations for reaction sequences (a), (b) and (c) (Scheme 2)

E = Fe-Mo-protein and e its concentration; M = Fe-protein and m its concentration; S = substrate (N_2 or C_2H_2) and s its concentration.

factors, unless they also are activated by the same treatments.

The meaning of K'

Silverstein & Bulen (1970) studied ATP-dependent H_2 evolution by the *Azotobacter vinelandii* nitrogenase complex and concluded that the two enzyme components, although not separated in their work, behaved as subunits in a reaction sequence (Scheme 1).

However, our results suggest that at least two Fe-protein molecules are involved in the sequence for each Fe-Mo-protein molecule in soya-bean bacteroid nitrogenase because of interaction between Fe-protein sites (Fig. 4). Also, reducible substrates may react at points 1, 2 or 3 in the sequence (Scheme 1). The further observation that the Fe-Mo-protein of *Clostridium pasteurianum* nitrogenase consists of two subunits (Dalton *et al.*, 1971), increases the possible number of steps in the sequence under some experimental conditions.

We have considered the following three possible reaction sequences in the experiments presented above, in which non-limiting ATP and reductant concentrations were used to estimate K' and V' with constant Fe-Mo-protein and increasing Fe-protein concentrations. The Fe-Mo-protein is regarded as the enzyme (E), the Fe-protein as a catalytic effector (modifier, M) and N_2 or C_2H_2 as substrate (S) (Scheme 2). These sequences were evaluated by the method of King & Altman (1956), using appropriate equilibrium diagrams. These diagrams and the simplified derived rate-equations are given in Fig. 10. In all three sequences, the rate constants of the product-forming steps are considered to contain undiscerned rate-constants involving ATP and reductant.

All three mechanisms give rise to expressions in which K' and V' change in value with increasing concentrations of Fe-protein (M) (Fig. 10) and in which K' and V' contain different combinations of the various rate constants. In our experiments (Fig. 3), K' and V' were positively correlated and the intercepts K'_0 obtained by extrapolations ($8.7\mu M-N_2$ and $10.9\mu M-C_2H_2$) were similar to those obtained from experiments in which ATP concentration produced concomitant variation in K' and V' ($10.6\mu M-N_2$ and $6.7\mu M-C_2H_2$; Fig. 6) and from experiments with intact systems (6.7 and $8.3\mu M-N_2$; Fig. 8). Understanding of the significance of the apparent consistency of these intercepts depends upon the establishment of the order of the reaction sequence. The theoretical treatments of the data considered so far, have assumed that S and M react before ATP. The observation that K_i for the competitive inhibitor CO was not affected by Fe-protein concentration (Fig. 3) supports S being the first reactant [Scheme 2(a)]. However, it is possible that ATP

reacts first. We conclude that more critical evaluation of the kinetics of nitrogenase-mediated reactions must await the establishment of the order in which the components of the system react, and accurate establishment of the stoichiometry of the various steps. In the meantime, we suggest that K'_0 is a more reliable parameter on which to base comparisons of nitrogenases from different sources, than the apparent Michaelis constant (K') which may vary with the conditions imposed in the test.

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