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KINETICS OF ACETYLENE AND CN^- REDUCTION BY THE N_2-FIXING SYSTEM OF *RHIZOBIUM LUPINI*

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SUMMARY

1. N_2-fixing extracts have been successfully isolated and partly purified from bacteroids of *Rhizobium lupini* obtained from yellow lupin nodules. These extracts catalysed acetylene and CN^- reduction, demonstrating the same substrate versatility as nitrogenase from other organisms.

2. The enzyme saturation curves by ATP for both acetylene and CN^- reduction were closely similar. These indicate that nitrogenase from this source has several sites for binding ATP. Intermediary plateaus suggest that cooperation of these sites involves both positive and negative changes in either binding or catalytic activity as the enzyme is saturated with ATP.

3. Curves for inhibition of acetylene and CN^- reduction by ADP also displayed mixed positive and negative cooperativity. It is proposed that ADP has an inverse effect to ATP on the rate of reductive catalysis by nitrogenase.

4. The complex kinetics displayed are consistent with a hypothesis that one aspect of the role of ATP is to induce conformational changes in nitrogenase. Also, the kinetics provide a possible mechanism for nitrogenase regulation by the available energy supply.

5. Tests with a variety of nitrogenous products of fixation and other compounds failed to provide convincing evidence for feed-back inhibition of the nitrogenase in *Rhizobium lupini*.

INTRODUCTION

It was shown previously for excised leguminous root nodules containing *Rhizobium lupini* as the microsymbiont, that ammonia was the primary uncombined nitrogen compound formed from $^{15}N_2$ (ref. 1). Amino acids such as glutamine and glumatic acid were then synthesised rapidly from ammonia, with other amino acids such as asparagine labelled in a manner suggesting them to be end points for N_2 fixed within these nodules, before transport to other plant tissues. Earlier studies[2,3] with soybean nodules containing *Rhizobium japonicum* as the microsymbiont produced qualitatively similar patterns of labelling. More recently, Rhizobium bacteroids isolated from soybean nodules[4,5] have been shown to contain the N_2-fixing system. The enzyme in this organism[6] is similar to that of the non-symbiotic organisms[7],

Clostridium and Azotobacter, composed of at least two metalloproteins which are O_2-labile and capable of reducing alternative substrates such as acetylene[5]. As with nitrogenase from all sources so far examined[7], there is an obligatory ATP requirement for substrate reduction to occur. At present, insufficient information is available to decide the precise role of ATP in biological N_2 fixation and also whether feed-back kinetic control of nitrogenase activity by the primary or tertiary products of fixation referred to above occurs.

In this paper it is confirmed that bacteroids of *Rhizobium lupini* contain nitrogenase, indicated earlier by labelling with $^{15}N_2$ (ref. 8) and that the enzyme system appears basically similar to that of *Rhizobium japonicum*. In particular, kinetic evidence is given that a mechanism exists for regulation of nitrogenase activity in lupin nodules by the energy supply at the site of fixation. On the other hand, no clear evidence for feed-back control of nitrogenase by nitrogenous products of fixation has been found.

METHODS

Growth of lupin nodules

Lupin (*Lupinus luteus* L. cv. Weiko III) nodules were collected from field-grown plants at or prior to flowering, frozen in liquid N_2 and stored at −15°. The WU425 strain of *Rhizobium lupini*[9] was used for inoculation. Since the crown nodules encircling the main root were used, it is unlikely that formation of nodules by other strains of soil rhizobia occurred[10].

Isolation of active bacteroids

Acetylene reduction[11,12] was employed as the criterion of activity during nitrogenase preparation. It was possible to obtain active extracts from nodules held for several days at 0°, or several months following rapid freezing in liquid N_2 (ref. 6) with subsequent storage at −15°.

Bacteroids were separated from plant material by homogenisation for 1 min (M.S.E. Atomix) of 100 g nodules with 150 ml of de-aerated 0.05 M KH_2PO_4–KOH buffer (pH 7) containing 0.2 M sucrose and 2 % (w/v) soluble polyvinylpyrrolidinone[5] (Kollidon-25, BASF). The homogeniser was fitted with gassing vents to displace air by a flow of N_2 during this operation. Large plant fragments were removed by filtration through cheesecloth under a stream of N_2 and the nodule-brei centrifuged for 10 min at 10000 × *g* to sediment the bacteroids. The pellet was washed once under N_2 by suspension in 10 times by volume of the buffer used above and re-centrifuged. Bacteroids prepared in this way reduced 0.05 atm acetylene to ethylene at variable rates in the range 5–25 nmoles/mg bacteroid protein per min with 50 mM sodium-succinate added. The optimum partial pressure of O_2 was 0.20 atm (*cf.* ref. 4) with the balance Ar. At 0.20 atm O_2, inactivation of the acetylene-reducing system, presumably by O_2, began after 40–60 min. No acetylene reduction was obtained with an initial O_2 pressure of 0.50 atm.

Preparation of nitrogenase extracts

Cell-free extracts were prepared by sonication of a 20 % (v/v) suspension of bacteroids in 0.05 M Tris buffer (pH 8.2). $Na_2S_2O_4$ (0.5 mM) was added with a gas-tight

syringe prior to sonication with all operations being performed under N_2 to prevent inactivation of the system by O_2. At each stage, suspensions and extracts were thoroughly degassed by repeated evacuation and flushed with Ar to maintain strict anaerobiosis. The cells were broken at 0–5° by 150-sec sonication in 5-sec bursts using full power of 125 W (Biosonik II, Bronwill Scientific). The sonicate was centrifuged at 25000 × *g* for 20 min at 5° and the supernatant, stored under Ar, used for enzyme assays. The extracts retained full activity (10–30 nmoles acetylene reduced per min per mg protein) for several weeks when frozen in a solid CO_2–ethanol mixture and stored at −15°. Extracts were desalted on anaerobic columns of Sephadex G-25 equilibrated with 0.05 M Tris–HCl (pH 8.2).

Partial purification

Purification was achieved by minor modifications of previously published methods[6,13]. Acidic proteins were adsorbed on DEAE-cellulose equilibrated with 0.05 M Tris–HCl (pH 8.2). The column was washed with 0.05 M Tris containing 0.1 M NaCl and the nitrogenase components eluted in a batch with 0.5 M NaCl in the Tris buffer. This eluate was diluted with 0.05 M Tris buffer to reduce the salt concentration to less than 0.05 M NaCl and reconcentrated under N_2 in an ultrafiltration cell (Diaflo, Amicon Industries). Each solution used contained 0.5 mM $Na_2S_2O_4$ to prevent oxidation. These preparations, purified approx. 4-fold with 50 % recovery, based on acetylene-reducing activity, supported reduction of N_2 to ammonia when assayed by methods described previously[14].

Separation of the nitrogenase into two brown-coloured components was achieved by filtration of the DEAE-cellulose extracts on anaerobic columns of Sephadex G-100. However, insufficient amounts of this more highly purified material were obtained to be used for the experiments described here. Examination by atomic absorption spectrophotometry of the two protein components obtained from the Sephadex G-100 columns showed that both contained iron, similar to the nitrogenase components prepared previously[6,13,15]. Both components were required for acetylene reduction.

Assays

Nitrogenase activity of the extracts was assayed by measuring the rates of CN^- (ref. 16) and acetylene[5] reduction. These were linear for 4–8 min following a short lag period. The gaseous products, methane and ethylene, respectively, were analysed by gas chromatography. Substrate levels of ATP were employed[14], with $Na_2S_2O_4$ as the electron donor[15]. The initial rates of CN^- and acetylene reduction were the same with substrate quantities of ATP as with an ATP-generating system, using creatine phosphate and creatine kinase (ATP:creatine phosphotransferase, EC 2.7.3.2).

The standard reaction mixture contained 50 μmoles TES (*N*-Tris(hydroxymethyl)methyl-2-aminoethane sulphonic acid)–KOH buffer (pH 6.8); 25 μmoles $Na_2S_2O_4$; 5 μmoles KCN or 0.25 ml acetylene; ATP and $MgCl_2$, as indicated; *plus* enzyme extract and water to 1.0 ml. Assays were carried out in 4.5 ml serum bottles fitted with rubber stoppers (A. H. Thomas, Philadelphia) which were thoroughly degassed and flushed with high-purity Ar prior to addition of $Na_2S_2O_4$ solution. Reaction was initiated by addition of enzyme extract following 5-min equilibration of the mixture at 30°. Tests showed that the concentrations of acetylene and CN^- used were saturating. Protein was determined with the biuret reagent.

Gas chromatography

Samples for gas chromatography were taken from each reaction vial at 1–2-min intervals and analysed using a 10-inch column of Porapak T (Waters Assoc. Mass.) with flame ionization detection (Perkin–Elmer F11). At 65°, both methane and ethylene were eluted from the column in less than 15 sec.

RESULTS

Response to ATP concentration

The response to ATP concentration by both acetylene reduction and CN^- reduction was sigmoidal (Fig. 1). As with all nitrogenase preparations so far examined, Mg^{2+} was needed with ATP, but as shown in the figure, no significant difference between ATP/$MgCl_2$ ratios of 0.5 and 2.0 in either total activity or in kinetic response was obtained.

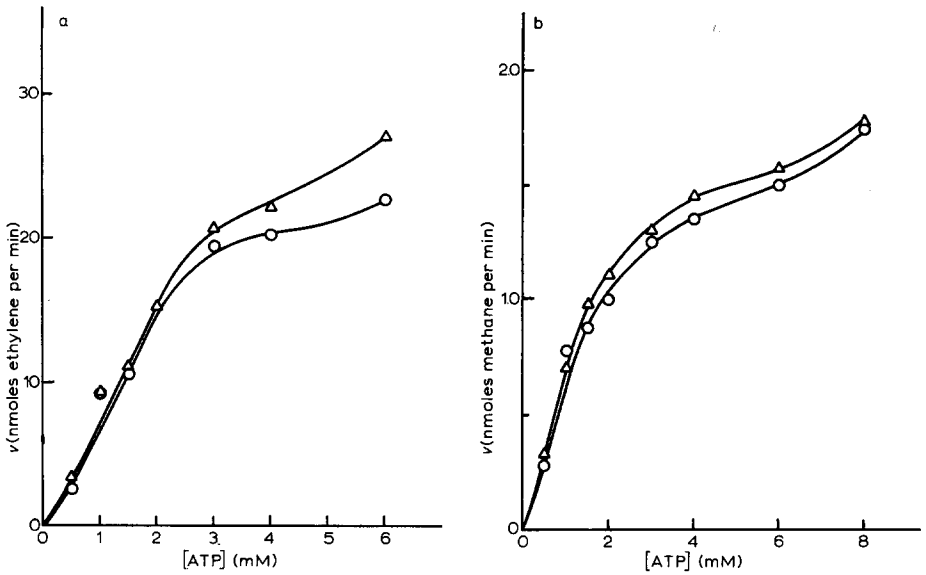

Fig. 1. Response curves to ATP concentration, a, acetylene reduction; b, CN^- reduction. Reaction mixtures were as described in METHODS, with 0.6 mg of protein (DEAE-cellulose extract) and ATP/$MgCl_2$ of 0.5 (○—○) and 2.0 (△—△).

It was possible that the sigmoid nature of the response curve at low ATP concentration was a consequence of inadequate Mg^{2+} rather than low ATP concentration. However, no difference in the sigmoid response of the rate of acetylene reduction by reaction mixtures containing a constant excess of 2 mM Mg^{2+} above ATP concentration compared to reaction mixtures with the constant ratio of ATP/$MgCl_2$ of 0.5 was observed, ruling out this objection.

More detailed analysis of the response curves obtained revealed that both acetylene and CN^- reduction by these N_2-fixing extracts did not conform to classical[17] allosteric kinetics. Although the inverse plots of $1/v$ *vs.* 1/ATP were concave upwards as expected for cases of positive cooperativity between two or more ATP-binding sites

on the enzyme, where the binding of one substrate molecule increases the ease of binding of the next, Hill plots[17] of log $(v/(v_{max} - v))$ *vs.* log [ATP] were not linear over the range of ATP concentrations employed. Moreover, the slopes of the linear portions of these plots (0.8–1.3 for the data in Fig. 1) indicated only slight cooperativity.

An explanation for this apparent anomaly is given by the results of the experiment shown in Fig. 2, where a distinct plateau in the curve for rate of acetylene reduction in the region of 2–5 mM ATP is shown. The occurrence and significance of such intermediary plateaus in enzyme saturation curves has been commented on by TEIPEL AND KOSHLAND[18]. Similar plateaus were obtained for CN^- reduction by these enzyme preparations, although the region of the plateau varied slightly from one enzyme preparation to another, independently of the stage of enzyme purification. These intermediary plateaus were easily overlooked when insufficient substrate concentrations were employed (see Fig. 1). Nevertheless, it was possible to diagnose the

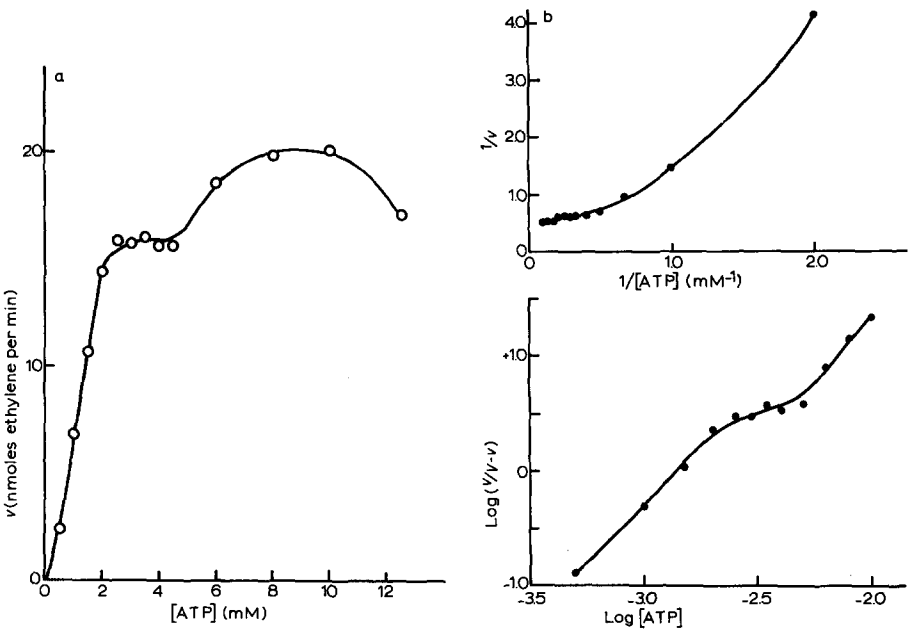

Fig. 2. Dependence of acetylene reduction on ATP concentration. The intermediary plateau lies in the region 2–5 mM ATP. Double reciprocal and Hill plots are shown in (b). ATP/$MgCl_2$ = 2.0, 1.3 mg unpurified extract.

deviation from classical allosteric kinetics from the Hill plots, which had linear regions with a slope about 1.0 and showed a significant increase in slope to about 2 at the extreme ATP concentrations, similar to that in Fig. 2(b). This type of deviation has been predicted to occur in cases of mixed positive and negative cooperativity[19].

Rates of CN^- and acetylene reduction

The relative rates of acetylene reduction and CN^- reduction were of the order 20 : 1, not 6 : 1 as would be anticipated for electron transfer forming ethylene on a specific binding site at the same rate as electron transfer for CN^- reduction to methane *plus* ammonia at the same substrate-binding site. This discrepancy can be partly

attributed to ATP-dependent formation of additional gaseous products from CN^-, since at least two other peaks with longer retention times were observed during gas chromatography. Small quantities of reduction products other than methane *plus* ammonia from CN^- have been observed with previous nitrogenase preparations[20,21]. It is also possible that ATP-dependent H_2 evolution by nitrogenase contributed to the imbalance of electrons consumed for ethylene production compared to methane formation, although this point was not examined in this study.

ADP inhibition

The effect of added ADP on the response curve to ATP concentration is shown in Fig. 3. For both acetylene and CN^- reduction, ADP apparently acted as a negative

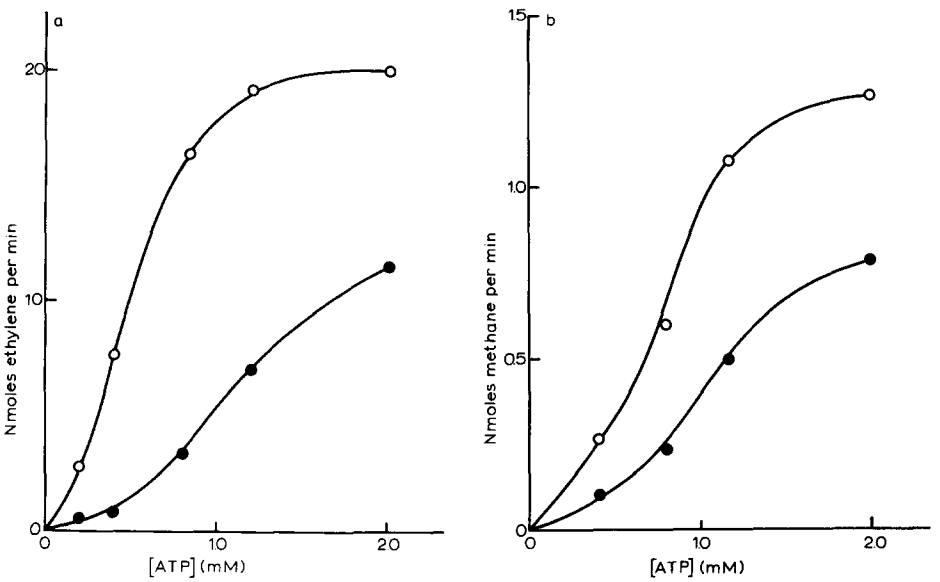

Fig. 3. Effect of ADP on response curves to ATP for (a) acetylene reduction: 0.4 mM ADP, 1.2 mg unpurified extract; (b) CN^- reduction: 0.8 mM ADP, 1.3 mg unpurified extract. ATP/$MgCl_2$ = 0.5. ○—○, *minus* ADP; ●—●, *plus* ADP. Analysis in duplicate; S.D. = 0.8 nmole acetylene and 0.03 nmole methane, respectively.

modifier on enzyme activity. Hill coefficients calculated for the range 0–2 mM ATP increased on addition of ADP from 2.1 to 2.7 for CN^- reduction and from 2.3 to 3.1 for acetylene reduction. The validity of such plots in indicating the number of binding sites is questionable, since the apparent v_{max} represents a plateau in the enzyme saturation curve, rather than a true maximum rate of activity. Nevertheless, the plots were linear, providing a useful index of the degree of positive cooperativity within this range.

For experiments in which the level of ATP was held constant and ADP concentration varied, curves of the type depicted in Fig. 4 were obtained. The concentration of ADP required for 50 % inhibition of enzyme activity was about 1/10 the ATP concentration employed. This degree of inhibition contrasts with results obtained for ADP inhibition of acetylene reduction[22], CN^- reduction[23], H_2 evolution and N_2

reduction[14] with nitrogenase from Clostridium and CN^- or isocyanide reduction by nitrogenase from Azotobacter[24], where 50 % inhibition required ADP/ATP ratios of approx. 1 : 2.

As shown in Fig. 4, with an initial saturating concentration of 10 mM ATP, the inhibition curve in the range 0–1.2 mM ADP also contained intermediary plateau regions. These were basically similar for both acetylene and CN^- reduction and produced non-linear Hill plots in this range with slopes varying from −0.3 at low concentrations of ADP to −1.8 at higher concentrations of ADP. Thus ADP inhibition of CN^- and acetylene reduction appears to be an inverse situation to activation by ATP.

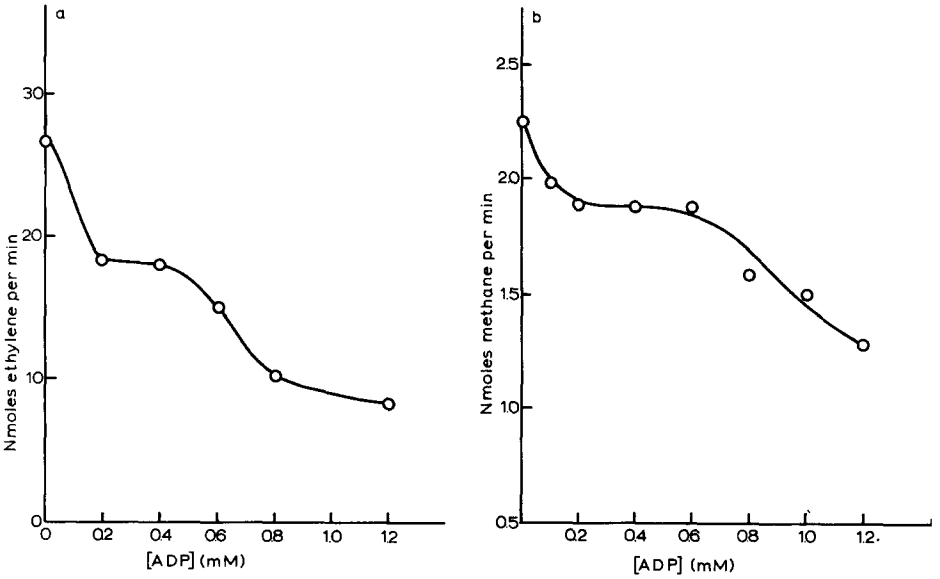

Fig. 4. Inhibition of acetylene (a) and CN^- (b) reduction by ADP with constant ATP (10 mM). (a) 0.8 mg protein *ex* DEAE-cellulose (b) 0.4 mg *ex* DEAE-cellulose: $ATP/MgCl_2 = 0.5$. Agreement between duplicate analysis was good; S.D. = 0.9 nmole acetylene and 0.02 nmole methane.

Effect of amino compounds and salts

Tests for responses to nitrogenous products of N_2 fixation indicated only small kinetic effects on enzyme activity at moderate concentrations up to 10 mM. Compounds tested included ammonia, glutamic acid, glutamine, aspartic acid, asparagine, alanine, glycine and γ-aminobutyric acid, which have been shown to be labelled in the initial 15 min during N_2 fixation *in vivo*[1], as well as histidine, tryptophan, leucine, valine, arginine, lysine, β-cyanoalanine, cysteine and urea. Sodium thioglycollate at 10 mM concentration inhibited activity 90 % and rapidly inactivated the system, although cysteine was not inhibitory.

Concentrations of ammonia or amino acids up to 50 mM or greater were required to elicit substantial inhibition of enzyme activity. However, similar or even greater inhibition was obtained at this concentration with salts such as K_2SO_4, $(NH_4)_2SO_4$ or KCl.

DISCUSSION

In these studies, acetylene and CN^- were chosen as substrates instead of N_2 because of the extreme sensitivity with which their reduction products can be measured. Although there have been at least two reports[14,26] of ammonia formation using substrate levels of ATP without resorting to an ATP-generating system such as creatine phosphate–creatine kinase, the reaction mixtures required to obtain sufficient ammonia for accuracy would be too large, particularly if the initial linear period of enzyme activity was to be measured.

The question arises as to whether the results reported here for acetylene and CN^- reduction can be extrapolated to N_2 reduction by the N_2-fixing system. Clearly this cannot be answered until a method of adequate sensitivity for assaying ammonia is developed, however, it is suggested that they can, since inhibition by ADP, the product from ATP utilization during nitrogenase activity[14], is a characteristic common to acetylene[22], CN^-(ref.23) and N_2 reduction[14] by the nitrogenase from Clostridium. Moreover, the assumption that the kinetics would also apply for N_2 reduction is consistent with current explanations of the kinetics of enzymes composed of subunits, in which cooperative effects induced by substrates and activators or inhibitors are attributed to changes in the conformation of the enzyme, altering the binding or catalytic capacity of active centres[17,18].

From the results reported, it seems likely that reduction of acetylene and CN^- with the partly purified enzyme used involves the cooperation of more than two ATP-binding sites despite Hill coefficients as low as 0.8 for uninhibited nitrogenase, since it is clear that the Hill equation as proposed in the concerted transition model of MONOD *et al.*[17] does not apply. Equations of the type proposed by TEIPEL AND KOSHLAND[18] and LEVITZKI AND KOSHLAND[19] are more satisfactory descriptions of the saturation curves observed. Even so, additional information on the extent and nature of subunit interactions in each functional nitrogenase unit will be needed before these kinetics can be explained. For instance, it is feasible that the saturation curves with intermediary plateaus result from several sub-unit associations of nitrogenase acting independently, each with different kinetics but catalysing the same reaction.

The kinetic complexity may be significant in explaining the role of ATP in N_2 fixation. One suggestion[18], to account for complex kinetics with positive and negative alternations in the enzyme saturation curve, is that induced conformational changes within the enzyme on ligand binding are rate-limiting rather than the catalytic processes which follow. Thus the results reported would agree with a hypothesis[25] that ATP-induced conformational changes in nitrogenase take place prior to substrate reduction. This also concurs with the following previous observations: (1) ATP utilization by nitrogenase isolated from Clostridium does not require that the site occupied by N_2 or alternative substrates be filled. The same rate of ATP utilization occurs whether a reducible substrate is present or not[26]. Therefore ATP utilization appears to be the rate-determining process in nitrogenase activity. (2) Breakdown of ATP by purified clostridial nitrogenase does not always require continous simultaneous donation of electrons to a reducible site (or sites) on the enzyme complex. Although an electron donor such as $Na_2S_2O_4$ stimulated ATP breakdown at neutral pH some 2–3-fold (I. R. KENNEDY and L. E. MORTENSON, unpublished results), results of BUI AND MORTENSON[27] showed that at a pH of 5.6 the same rate of P_i release occurred whether

$Na_2S_2O_4$ was present or not and the ATP saturation curves obtained had characteristics of curves with intermediary plateaus.

Conformational changes may generate reduced chemical species on the enzyme with greater reactive potential, comparable to the conformational states which have been proposed to accompany the electron transfer-mediated synthesis of ATP from ADP and P_i within biological organelles such as mitochondria and chloroplasts[28,29]. ATP-supported electron transfer for N_2 reduction by nitrogenase is at least formally the reverse reaction. Further studies to test this hypothesis are clearly required, preferably employing techniques other than enzyme kinetics.

The kinetics observed also provide a possible mechanism for regulation of the nitrogenase in lupin nodules. Advantages of saturation curves displaying negative cooperativity for control of enzyme activity have been explained[30]. In regions of positive cooperativity, small changes in the concentration of ATP would lead to relatively large changes in enzyme activity. Conversely, in regions of negative cooperativity, the enzyme system would be relatively unresponsive to changes of ATP concentration within this region. The inhibition by ADP, which also possesses characteristics of mixed positive and negative cooperativity, provides similar possibilities for regulation of the enzyme activity by product inhibition of the system.

Tests to show that nitrogenase could be subject to feed-back inhibition by nitrogenous products of fixation, in order to conserve energy reserves within the nodule, have been inconclusive. The inhibitions observed could be equally well explained as non-specific ionic effects. Also, products of N_2 fixation in nodules might control the rate of N_2 fixation at points remote from the nitrogenase enzyme, such as on the system for transport of electrons to nitrogenase, or on the ATP-generating system. For instance, inhibition of β-hydroxybutyrate dehydrogenase by glycine has been reported for soybean bacteroids[31]. β-Hydroxybutyrate is one of several potential electron donors for N_2 reduction in soybean nodules[32].

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