Review Letter

NITROGEN FIXATION AND BIOENERGETICS: THE ROLE OF ATP IN NITROGENASE CATALYSIS

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Received 3 November 1978

1. Introduction

Nitrogenase, the enzyme system responsible for biological nitrogen fixation, is found only among a minority of the procaryotic organisms, but the process it catalyzes is nevertheless of great importance for life on earth. The research efforts in this multidisciplinary area are enjoying a vigorous growth in interest [1–5], in part due to developments in the world's food and energy supplies. Industrial nitrogen fertilizers are energy-costly, and although this is also true for biological nitrogen fixation, the organisms involved use solar energy directly without intervening steps of technology.

Major research efforts on the biochemistry of nitrogen fixation are justified by our curiosity on how enzyme proteins can carry out the difficult (for the chemist) process of reducing dinitrogen to ammonia under such mild conditions. The research is also justified by the need to define the biochemical requirements for nitrogen fixation before work in genetics, ecology and agronomy can be realistically expected to increase the number of useful nitrogen-fixing systems. Our understanding of nitrogenase structure and function has progressed far since the first report on a method for preparation of cell-free extracts with vigorous nitrogen fixation activity in 1960 [6]. Reviews on the different aspects of nitrogenase research are available [1–5,7–10], and this Review Letter will concentrate on one aspect: The mechanism of energy coupling in nitrogenase catalysis, whereas the other aspects will be covered only briefly. Nitrogenase carries out ATP hydrolysis coupled with electron transfer [11–14], and the process can be studied with pure enzyme proteins in simple and well-defined media. The rapidly-increasing understanding of this system should be relevant to our general concepts of bioenergetics, including the more complex systems of oxidative phosphorylation and photophosphorylation.

2. Nitrogenase and its reactions

The nitrogenase proteins have been purified completely or partially from a variety of different organisms. The evidence thus far suggests a basic similarity in the requirements for this enzyme reaction: Two proteins, the Fe protein and the MoFe protein of nitrogenase; a low potential reductant; ATP and Mg^{2+}. The properties of the nitrogenases from different organisms are rather similar with regard to molecular weight, subunit composition, metal content, spectroscopic properties and kinetics. The observed species differences are compatible with one common mechanism, but of course there are minor structural and kinetic variations due to evolution.

The nitrogenase proteins must interact during catalysis and form a complex. Because the complex dissociates during each catalytic cycle, a new nomenclature: *Nitrogenase reductase* for the Fe protein and *nitrogenase* for the MoFe protein, has been proposed [15]. Although this is a logical nomenclature, this review will use the more established terminology of denoting the enzyme system of both proteins as nitrogenase.

Despite their necessary affinity for each other, the

two proteins can be completely separated, and they can be obtained free of other proteins and membrane fragments by standard techniques. This is also true for the nitrogenase proteins of *Azotobacter vinelandii* [16], even though much work has been done with a particulate form of the nitrogenase of this organism [17]. The purification of active nitrogenase proteins is straightforward, except for one feature: Both proteins (the Fe protein to the worst extent) are rapidly inactivated by O_2, and they must always be handled under strictly anaerobic conditions. Anaerobic techniques are necessarily more cumbersome than the more familiar aerobic work and limits the number of experiments performed per nitrogenase enzymologist. Another problem is that the presence of unknown proportions of O_2-inactivated proteins introduces ambiguity in the interpretations. Nevertheless, these problems can be overcome without resort to heroic efforts.

The MoFe proteins have molecular weights around 220 000 and consist of 4 subunits; these are of two different types in most if not all MoFe proteins [18–20]. They contain 20–30 iron atoms, 20–30 acid-labile sulfur atoms, and 1–2 molybdenum atoms per MoFe protein molecule of 4 subunits. The EPR spectrum of the MoFe protein is characteristic [21–23] and does not resemble EPR spectra of simpler iron–sulfur proteins, indicating either spin-coupling between several iron–sulfur clusters, or iron–sulfur clusters of presently unknown structure, or both. The molybdenum does not contribute directly to the EPR spectrum [24].

A breakthrough in our understanding of the metal-sites and N_2-activating site in the MoFe protein is expected soon, as a consequence of the report by Shah and Brill [25] on a low-molecular weight prosthetic group of the MoFe protein, the iron–molybdenum cofactor. The complete structure of this molecule will hopefully soon be known; available information at present indicates a ratio of iron to acid-labile sulfur to molybdenum of 8:6:1. Approximately half of the iron and all of the molybdenum resides in the cofactor, which shows an EPR spectrum rather similar to that of the intact MoFe protein [26]. The remaining iron and sulfur show no EPR signals, but can be studied with Mössbauer spectroscopy.

The Fe protein is the smaller of the two proteins, 55 000–65 000 daltons, and consists of two identical subunits (confirmed by the complete amino acid sequence of the Fe protein from *Clostridium pasteurianum* [27]). Present consensus favors one Fe_4S_4 cluster (bacterial ferredoxin type) per Fe protein molecule of two subunits [10,28,29]. It is tempting to further speculate that the Fe_4S_4 cluster bridges two identical and symmetrically combined subunits, being anchored through two cysteines on each subunit. Direct evidence for this, however, has not been provided.

Nitrogenase catalyzes many different reactions, but a fundamental chemical equation is:

$$N_2 + 6\,e^- + 12\,MgATP + 8\,H^+ \rightarrow$$

$$2\,NH_4^+ + 12\,MgADP + 12\,P_i$$

Electron donors can be ferredoxins, flavodoxins or dithionite ion. ATP (not other nucleoside triphosphates) and a divalent metal ion, usually Mg^{2+}, are absolute requirements for any electron transfer reactions by nitrogenase; the observed number of ATP molecules consumed is often higher, but never lower than indicated.

Besides N_2, a number of other molecules and ions can be reduced through nitrogenase catalysis, e.g., N_2O, N_3^-, C_2H_2, HCN, CH_3NC, H^+. Of these, the reduction of acetylene to ethylene is of great practical importance for the measurement of nitrogenase activity in vivo [2] and the reduction of protons to dihydrogen is important because this substrate is always present with active nitrogenase systems. In fact, total electron transfer is as fast under argon (with H^+ as the only reducible substrate) as under N_2, and N_2 reduction under 1 atm N_2 is accompanied by some H_2 evolution [28,30,31]. Hence, the rate of activation and reduction of N_2 is not limiting for the earlier steps of electron transfer and energy coupling. This poses problems in designing experiments on the mechanism of electron transfer, because functioning nitrogenase can never be studied in the absence of reducible substrate.

Our present view of the sequence of electron transfer in nitrogenase, which derives mostly from experiments with EPR spectroscopy [21–23,32], is:

$$\text{Electron donor} \rightarrow \text{Fe protein} \rightarrow \text{MoFe protein} \rightarrow N_2$$

This fits well with the popular but unproven supposi-

tion that molybdenum is part of the N_2-reducing site. The Fe protein shows a ferredoxin-type EPR signal in its reduced state, and this signal disappears when the system of both nitrogenase proteins is allowed to exhaust the supply of reductant (dithionite). The MoFe protein exists in three EPR-defined oxidation states: An oxidized state, which is EPR silent, is formed by dye-oxidation, and is probably not part of the catalytic cycle; the 'native' state, which is observed in the presence of dithionite and shows the characteristic EPR spectrum; and the 'super-reduced' state, which is also EPR silent and is formed only through reduction by the Fe protein in the presence of ATP. The 'super-reduced' state returns to the 'native' state upon exhaustion of dithionite. An excellent review of the experiments and reasoning behind the scheme for electron flow is found in [10].

3. Binding of ATP to the Fe protein of nitrogenase

Bui and Mortenson used gel filtration to show that ATP binds to the Fe protein, but not to the MoFe protein [33]. Tso and Burris extended equilibrium binding studies to quantitative experiments with a gel equilibration technique [34]. They found that ATP binds to two independent and equivalent sites on the Fe protein from *C. pasteurianum* with a site-specific dissociation constant, K_s = 17 μM. ADP competes for binding at one site, but at the same time ADP increases the affinity of the other site for ATP. The dissociation constant for ADP is 5 μM.

Mg^{2+} (or certain other divalent metal ions) is necessary for binding of ATP to the Fe protein and for any enzymic activity of nitrogenase. The metal ions act by forming a complex with ATP, and it is this complex rather than free ATP that reacts [35]. Thus, when discussing effects of ATP, I refer to the complex between Mg^{2+} and ATP rather than free ATP, and the same applies to ADP as inhibitor.

The binding of ATP induces a change in the EPR spectrum of the Fe protein, from a rombic to an axial type [21–23]. Zumft et al. [36] titrated the Fe protein from *C. pasteurianum* with ATP using EPR and found that 2 ATP per Fe protein was necessary for complete conversion of the EPR spectrum. The concentration of Fe protein was high (~0.3 mM) in these titrations, and a reasonably sharp break in the titration curve was expected, allowing determination of the stoichiometry, but not the dissociation constant or the type of binding curve. Smith et al. [23] in similar experiments with the Fe protein from *Klebsiella pneumoniae* observed weaker binding and indicated a dissociation constant of 0.4 mM, assuming a hyperbolic relationship between ATP concentration and the proportion of the Fe protein in the ATP form. The possibility of sigmoid rather than hyperbolic behavior due to the possible need for simultaneous binding to both ATP sites was not considered.

An attractive hypothesis originating from the EPR shift is that the binding of ATP to the Fe protein leads to a conformational change which alters the environment of the Fe_4S_4 cluster and allows for energy coupling during electron transfer from this group to some group on the MoFe protein. Interestingly enough, binding of ATP lowers the redox potential of the Fe protein from –0.29 V to –0.40 V [10,37]. These measurements were made at equilibrium, and there is much more energy available from ATP hydrolysis in turnover. Nevertheless, we may consider the potential shift a prelude to an energy transfer step which requires hydrolysis of the ATP and only takes place in the complex of the component proteins.

A third line of evidence for interaction between the ATP sites and the iron–sulfur site on the Fe protein was provided by Walker and Mortenson [38]. They discovered that ATP dramatically increases the rate of reaction between native Fe protein and the iron chelator α,α'-dipyridyl; Mg^{2+} is required for this effect, ADP inhibits, and other nucleoside triphosphates have little or no effect. The MoFe protein shows no such response to ATP. Again, conformational changes caused by ATP binding were suggested.

ATP-binding to the Fe protein probably exposes the Fe_4S_4 cluster to solvent, a conclusion drawn for the following reasons:

(i) The EPR shift with ATP is similar to the shift with 5 M urea, a denaturant that most likely exposes the iron–sulfur site [36];

(ii) Increased rate of chelation seems consistent with increased exposure, as ferredoxins increase their chelation rates upon denaturation [39];

(iii) The decrease in the redox potential is a shift in the expected direction when the Fe_4S_4 cluster is moved from the interior to the exterior of the protein, judging from the trends observed with synthetic iron–sulfur model compounds [40].

Kinetic studies [41] of the ATP-induced chelation reaction with bathophenanthrolinedisulfonate supported the rate equation: $v/V_{max} = ([ATP]/(K_s + [ATP]))^2$. This equation describes a sigmoid curve, but a hyperbola when plotted as $v^{1/2}$ versus [ATP]. Hence, the kinetics of chelation is consistent with a model in which both of the two ATP sites must be occupied to allow the conformational change that increases the reactivity of the iron–sulfur site. The alternative model, with the increase in reactivity being proportional to the total number of occupied ATP sites, predicts a hyperbolic rate equation and was not supported by the experimental data. The conclusion is that sigmoid relationships are not due to interaction between the two binding sites for ATP, but to interaction with a third and different site: The iron–sulfur site, and to the requirement that both binding sites must be occupied before this interaction takes place.

Similar analysis of the coupling as it is observed in the ATP-induced shifts of EPR and redox potential have not yet been presented. Hopefully, such endeavors will be undertaken, even in the face of greater experimental obstacles than for the chelation experiments. The previously-mentioned titration experiments at high Fe protein levels [36] indicate that both ATP sites are involved in the EPR shift, but a choice between the possible models for coupling cannot be made from available data. Certainly, models analogous to that proposed for the chelation reaction [41] must be kept in mind.

4. Stoichiometry and kinetics

The number of ATP molecules hydrolyzed per electron transferred (ATP/e^-) during nitrogenase catalysis is variable. Values of 10 or more have been observed with no apparent upper limit, but a lower limit of 2 is indicated [42–45]. The implication is that ATP hydrolysis can be uncoupled from electron transfer, but not vice versa, and that the stoichiometry during tight coupling is 2 ATP/e^-. This stoichiometry is also supported by the binding and kinetic studies with ATP [34,41], which are consistent with ATP hydrolysis at two sites coupled with the transfer of one electron [46] from the single iron–sulfur site on the Fe protein.

Experimental conditions that lead to high ATP/e^- ratios are temperatures higher or lower than 20°C [43,45], excess MoFe protein [44], or heterologous combinations of component proteins [47,48]. The temperature effect is most pronounced below 20°C, suggesting that at lower temperatures one or both of the nitrogenase proteins is changed into a conformation that is inactive in electron transfer, but is still capable of ATP hydrolysis [45]. Some experimental support for this view comes from the observation [41] that the reactivity of the Fe protein towards bathophenanthrolinedisulfonate in the absence of ATP increases with decreasing temperature to 0°C. This suggests a rapid, reversible conformational change which is distinct from the slow cold inactivation of the Fe protein from *C. pasteurianum*. No similar effect was seen with the MoFe protein.

The high ATP consumption during nitrogen fixation appears wasteful of energy, and the question arises whether it is an artifact of cell-free extracts. The answer seems to be no however, as whole-cell nitrogen fixation also consumes large amounts of ATP [49–51]. Thus, we are forced to conclude that biological nitrogen fixation is inherently energy-costly.

Kinetic studies of ATP utilization by nitrogenase are complicated by the rapid onset of product inhibition by ADP [28,52]. Early claims for complicated, sigmoid kinetics at high ATP levels were based on experiments where uncontrolled product inhibition prevented the measurement of true initial velocities [53–55]. More careful measurement of initial velocities, in the presence of an ATP-generating system, revealed no obvious deviations from hyperbolic behavior in the range of ATP concentrations from the apparent Michaelis constant (0.1–0.4 mM) and upwards [28,35]. Nevertheless, at lower ATP levels the relationship becomes clearly sigmoid [56]. This is expected if electron transfer from the Fe protein requires ATP hydrolysis at both of the two ATP sites [41]. ADP blocks one of these sites and reveals the sigmoid character of the plots at higher ATP levels. Thus, there is no conflict between the observations of hyperbolic binding curves [34] and sigmoid kinetic curves. On the other hand, analysis of nitrogenase kinetics based on current models for allosteric behavior [57] is of doubtful value, because the assumptions needed for application of these equilibrium binding models to steady-state kinetics are not valid for nitrogenase [41].

As opposed to steady-state kinetics, stopped-flow spectrophotometric investigations of the ATP dependence of the pre-steady-state rate of electron flow from the Fe protein to the MoFe protein did not reveal sigmoid kinetics in the presence or absence of ADP [57]. This appears in conflict with the model of 2 ATP sites in coupling with 1 iron–sulfur site. Further investigations at low ATP levels will, however, be necessary to exclude the possibility of sigmoid pre-steady-state kinetics [41].

The inhibition by ADP provides a mechanism for regulation of nitrogen fixation [8,10,52,58], and this seems appropriate for such an energy-costly process. There is no need, however, to postulate special regulatory sites for ADP.

Another level of nitrogenase control is exerted by the photosynthetic bacterium *Rhodospirillum rubrum* [59]. Here an activating protein is needed to transform inactive Fe protein to an active form, and this activation step requires ATP without hydrolyzing it.

5. Why is ATP needed for nitrogenase catalysis?

The Gibbs free energy of formation of NH_4^+ from N_2 and H_2 is negative [60]. The energy of ATP hydrolysis is therefore not needed to drive an otherwise unfavorable equilibrium in the desired direction, but rather to overcome the high activation energy barrier for cleavage of the stable N≡N bond. Nitrogenase most certainly lowers the activation energy for N_2 reduction, but the remaining barrier is still so high that the reaction will only proceed with the input of extra energy. Or in other words, most enzymes find a way around the mountain of activation energy, but nitrogenase faces a range so high and rugged that even the lowest pass is too difficult to climb without a push from ATP.

The pathway and possible intermediates between N_2 and NH_4^+ are not well understood. Free intermediates are not formed by nitrogenase, but a pathway of stepwise 2 electron reductions with enzyme-bound intermediates has been proposed. Thus, the ATP requirement can be understood in light of the positive ΔG for reduction of N_2 by H_2 to N_2H_2 or N_2H_4, although the effect of the enzyme complexing the intermediates probably diminishes the free energy requirement [60].

I have argued for a model of two ATP molecules being hydrolyzed while one electron is transferred from the Fe_4S_4 cluster on the Fe protein onto the MoFe protein. Further speculation suggests that energy is conserved at this step by the reduction of a group on the MoFe protein with a lower redox potential than that of the donor group on the Fe protein. Thus, the energy released in ATP hydrolysis could be used for driving the electrons to a low potential, and subsequently, these highly reactive reduced groups would be able to overcome the activation energy barrier for N_2 reduction. Accordingly, nitrogenase carries out reductive dephosphorylation.

A calculation from the standard Gibbs free energy at pH 7 for hydrolysis to ADP suggests that 2 ATP/e^- lowers the potential by ~0.75 V [60] (or more at physiological levels of reactants). As the electrons are entering the system at approximately the level of the hydrogen electrode at pH 7, or somewhat lower [61] this means that with reasonably complete conservation of energy at this step, groups on the MoFe protein with mid-point potentials of −1.0 V or lower are reduced. No evidence on such low potentials has been provided so far. Nevertheless, several lines of circumstantial evidence are consistent with the proposal: The failure to reduce the MoFe protein (from the 'native' to the 'super-reduced' state) with anything but the complete system of Fe protein, ATP and reductant; the instability of reduced MoFe protein, i.e., rapid reduction of H^+ in the absence of other substrates; no inhibition of electron flow by 1 atm H_2 under conditions where H_2 is the product [28,43].

Other functions for ATP in nitrogenase catalysis have been proposed. Some of these proposals are alternatives to reductive dephosphorylation, some are complementary and some are both. Direct interaction between ATP and the substrate reduction site has been proposed, mainly on the basis of model chemistry [60,62]. These proposals have not received much support from studies of nitrogenase itself and appear in conflict with the evidence for the N_2-reducing site on the MoFe protein and the ATP sites on the Fe protein.

Smith et al. [47] studied the partially active heterologous nitrogenase system consisting of the Fe protein from *C. pasteurianum* and the MoFe protein from *K. pneumoniae*. Compared with the homologous enzymes, the steady-state activity of the heterologous

enzyme was lowered to 40% for ATP hydrolysis but to 12% for electron transfer, whereas the pre-steady-state rate of ATP-induced electron transfer from Fe protein to MoFe protein was unaffected [47,63]. Moreover, with homologous combinations, this initial electron transfer was completed in a time much shorter than the turnover time of the enzyme, even at ATP concentrations much lower than the apparent Michaelis constant. Smith et al. explained the apparent discrepancies by proposing that ATP is also involved in a stage in the enzymic reaction other than electron transfer between the two proteins, with one ATP site unaffected in the heterologous cross-reaction, but with another site being considerably perturbed and virtually uncoupled from the enzyme reaction.

The following lines of evidence suggest an alternative explanation for the results of Smith et al.:

(i) Hageman and Burris [15] showed that the complex dissociates after each electron is transferred between the component proteins, and in their studies of the mechanism of inhibition of electron transfer by excess MoFe protein they proved that the major route of electron transfer into the system is via free, oxidized Fe protein [64].

(ii) Emerich et al. [48] investigated the properties of the inactive complex between the Fe protein of *C. pasteurianum* and the MoFe protein of *A. vinelandii* and discovered that the two ATP sites on the Fe protein in the complex are accessible for binding of ATP, but the Fe_4S_4 cluster is not accessible to reaction with chelators in the presence of ATP. With one of these proteins as inhibitor of the other homologous nitrogenase, substrate reduction is inhibited completely, but ATP hydrolysis is inhibited only partially. The heterologous complex is much tighter than the homologous complexes [48,65].

Thus, the following model is consistent both with these observations and those of Smith et al.: The normal sequence of events is that the *free* Fe protein binds two molecules of ATP and accepts one electron, followed by binding to the MoFe protein, electron transfer, and ATP hydrolysis. Dissociation of the complex is required before the Fe protein accepts another electron. With the heterologous combinations, dissociation is probably slow, and this will affect the steady-state rate but not the rate of the initial electron transfer. On the other hand, steady-state ATP hydrolysis continues because the ATP sites are accessible in the complex. Excess homologous MoFe protein could act similarly by tying up oxidized Fe protein before the latter can accept electrons [64].

Rennie et al. [66] used antibodies as inhibitors and found that antibodies against the MoFe protein caused uncoupling of ATP hydrolysis from electron transfer under N_2 or argon, but not under acetylene. They suggested these observations as further evidence of multiple roles of ATP and also as evidence for an ATP site on the MoFe protein in the functioning complex. Alternative explanations seem feasible, however, in light of results showing that acetylene modifies nitrogenase and stimulates electron flow under certain conditions [67]. Thus, such effects of acetylene could lead to tighter coupling between ATP hydrolysis and electron transfer.

6. Conclusion

Our understanding of nitrogenase and its catalytic mechanism is improving rapidly. The experimental system is relatively simple, consisting of two purified proteins, but at the same time nitrogenase is a complex system of energy coupling; electron transfer and storage; and binding and activation of N_2. In this review I have favored reductive dephosphorylation over alternative roles for ATP. Perhaps this reveals a preoccupation with Occam's razor: The simpler model where ATP functions only in coupling with electron transfer from the Fe protein to the MoFe protein can explain available data and should therefore be preferred to more complicated models. But such a view must not prevent us from keeping multiple roles for ATP in mind.

Present knowledge on ATP and nitrogenase does not allow a detailed description of the molecular events. We do not know why the binding of ATP causes a conformational change, or how and when the hydrolysis step is coupled to the electron transfer step. Another question is the role of protons. Answers to these questions may also contribute to understanding of energy coupling in other biochemical systems. Nitrogenase has been included in discussions on electron-transport coupled ATP synthesis [68,69] and seems particularly relevant to conformational coupling models. The growing interest for nitrogenase as a

problem in bioenergetics is also indicated by a recent report on ATP synthesis by nitrogenase [70]. Such claims for reversal of the nitrogenase reaction are not entirely convincing, however, until more details on the reactions are known: Firstly, the ATP synthesis must be achieved with purified nitrogenase proteins to rule out the presence of other ATP-synthesizing enzymes, and secondly, the reversed electron transfer and the stoichiometry must be defined.

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