The role of MgATP in nitrogenase catalysis

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The role of MgATP in nitrogenase catalysis

Proefschrift

ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. C.C. Oosterlee,
in het openbaar te verdedigen
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van de Landbouwuniversiteit te Wageningen

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STELLINGEN

1. De experimentele resultaten van Reddy en Vaidyanathan rechtvaardigen niet de konklusie dat benzoate-4-hydroxylase tetrahydropteridine als cofaktor nodig heeft voor enzymatische aktiviteit.
   Reddy, C.C. & Vaidyanathan, C.S. (1975) Biochim. Biophys. Acta 348, 46-57.

2. Uit de door Tso en Burris beschreven bindingsexperimenten van ATP en ADP aan komponent 2 van nitrogenase uit Clostridium pasteurianum valt niet op te maken in welke oxidatietoestand het nitrogenase eiwit zich bevindt tijdens de gebruikte gel equilibratie methode.
   Tso, M-Y. & Burris, R.H. (1973) Biochim. Biophys. Acta 309, 263-270.

3. De konklusies die Hageman et al. trekken uit één enkel experiment, waarbij de hoeveelheid fosfaat, die vrijkomt tijdens de pre-steady-state hydrolyse van ATP door nitrogenase van Azotobacter vinelandii, bepaald werd, getuigen van "wishful thinking" en blijken bovendien onjuist te zijn.
   Hageman, R.V., Orme-Johnson, W.H. & Burris, R.H. (1980) Biochemistry 19, 2333-2342.
   Dit proefschrift.

4. Aktiviteitsmetingen van pteridine-afhankelijke mono-oxygenasen door bepaling van de oxydatiesnelheid van NAD(P)H in een assay waaraan een pteridine verbinding als cofaktor is toegevoegd zijn onbetrouwbaar.
   Shailubhai, K., Sahasrabudhe, S.R., Vora, K.A. & Modi, V.V. (1983) FEMS Microbiol. Lett. 18, 279-282.
   Kaufman, S. (1979) J. Biol. Chem. 254, 5150-5154.

5. Studies naar induktie, repressie en inhibitie van amylase door o.a. zetmeel en glucose bij hyfenvormende schimmels als Neurospora crassa en enkele Aspergillus soorten leveren zoveel verschillende en tevens tegenstrijdige konklusies op, dat het aanbeveling verdient de verschillende aspekten in één en dezelfde studie te analyseren.

6. De bewering dat met het huidige energieverbruik op korte termijn een tekort aan fossiele energiebronnen zal ontstaan, is gebaseerd op verkeerde schattingen van deze brandstofvoorraden en wordt dan ook ten onrechte als argument aangevoerd voor de noodzaak van kernenergie.

7. Bindingsstudies waarbij het maximale aantal bindingsplaatsen van een ligand op een eiwit bepaald wordt door gebruik te maken van ligand geïnduceerde spektrale veranderingen, zoals circulair dichroisme of ESR, van het betreffende eiwit zijn diskutabel.

   McKenna, C.E., Stephens, D.J., Eran, H., Luo, G.M., Matai Ding, F.X.Z. & Nguyen, H.T. (1984) in Advances in nitrogen fixation research (Veeger, C. & Newton, W.E., eds.) pp. 115-122, Nijhoff/Junk, Pudoc.

   Zumft, W.G., Palmer, G. & Mortenson, L.E. (1973) Biochim. Biophys. Acta 292, 413-421.

8. Nadat de EEG jarenlang de landbouwproduktie door subsidies heeft gestimuleerd, is het niet rechtvaardig de inkomenseffekten als gevolg van gedwongen produktiebeperkingen alleen tot het bedrijfsrisiko van de agrariër te rekenen.

9. In de talloze handboeken over enzymkinetiek worden in het algemeen dezelfde soort reaktiemechanismen beschreven. Het uitwerken van een reaktiemechanisme voor een enzym dat uit meerdere dissocieerbare eiwitkomponenten bestaat die samen nodig zijn voor enzymaktiviteit zou in dit opzicht vernieuwend zijn.

10. Men kan zich afvragen of het invoeren van de APK-autokeuring niet meer bijdraagt tot het rendement van garages dan tot de verkeersveiligheid.

11. Het blijkt overduidelijk uit de kwaliteitsvergelijking van de fietspaden en van de autowegen, dat de Nederlander pas bepaalde voorrechten geniet als er belasting voor betaald is.

Jan Cordewener

Wageningen, 7 mei 1987

Aan Marie-José en Kim

Aan mijn ouders

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Voorwoord

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Tot slot wil ik Jaap Visser noemen, die het mogelijk maakte dat ik tijdens mijn werkzaamheden op de vakgroep Erfelijkheidsleer dit proefschrift kon voltooien.

Contents

Abbreviations

ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
ATPase	ATP phosphohydrolase (E.C. 3.6.1.3)
BPS	bathophenantroline disulphonate
CD	circular dichroism
Da	dalton = mass of one hydrogen atom
DOC	deoxycholaat
DNA	deoxyribonucleic acid
EPR	electron paramagnetic resonance
F_1F_0	H^+-ATPase
Hepes	4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid
K	stoichiometric or macroscopic dissociation constant
K^i	intrinsic or microscopic dissociation constant
K_m	Michaelis-Menten constant
P_i	inorganic phosphate
SDS	sodium dodecyl sulfate
TCA	trichloroacetic acid
Tes	2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-amino}ethane sulfonic acid
$\Delta\tilde{\mu}_{H^+}$	proton-motive force
$\Delta\Psi$	transmembrane electrical potential
ΔpH	transmembrane pH gradient

The MoFe and Fe proteins of the nitrogenases of Azotobacter vinelandii, Clostridium pasteurianum, Klebsiella pneumoniae and Rhizobium japonicum are referred to as Av_1 and Av_2, Cp_1 and Cp_2, Kp_1 and Kp_2 and Rj_1 and Rj_2, respectively.

Chapter 1
Introduction

The four most abundant elements in living organisms are hydrogen, oxygen, carbon and nitrogen. Besides water, that makes up the bulk of the mass, most cells are mainly composed of proteins, nucleic acids, carbohydrates and lipids. All organic biomolecules of living organisms are ultimately mainly derived from very simple, low-molecular-weight precursors obtained from the environment, namely, carbon dioxide, water and dinitrogen plus in addition metals and phosphorus. Living organisms in nature continually recycle the chemical elements of which they are composed. The carbon and oxygen cycles may roughly be described as follows: photosynthetic cells, using solar energy, produce organic compounds such as glucose from atmospheric CO_2 and evolve dioxygen. Heterotrophic cells use dioxygen and glucose during respiration, thus returning carbon atoms to the air as CO_2. Nitrogen is another important element that cycles through living organisms. Two essential components of cells contain nitrogen, i.e. proteins and nucleic acids. Both nitrogen fixation -the conversion of dinitrogen to NH_3, a form which plants can use- and photosynthesis are key metabolic processes. They lead to the production of reduced nitrogen and carbon compounds, essential for the maintenance and continuation of life on earth.

1.1. The Nitrogen Cycle

The global nitrogen cycle can be drawn in either a simple or a complicated form as one wishes (see Fig. 1). The enormous quantity of elemental nitrogen associated with the earth may be roughly subdivided in three major sinks: in the gaseous form in the atmosphere (N_2, N_2O, NO_x, NH_3), in the oceans (plants, animals, microorganisms, soluble and suspended nitrogen compounds) and on land (soil, plants, animals, microorganisms). Terrestrial nitrogen is largely locked up in the earth's primary rocks, from which it is released extremely slowly as the surface weathers and soil formation occurs. It is estimated that the organisms of the soils and waters of this planet together contain about 1.5×10^{10} tonnes of N, while organic and inorganic matter of the soil carries about 3.3×10^{11} tonnes of N [1,2]. The greatest source of nitrogen for the biosphere is the atmosphere, a reservoir generally recorded as 3.9×10^{15} tonnes of N, mainly in the form of dinitrogen, N_2. Since the N_2 molecule is relatively inert chemically, it cannot be used by most organisms. It is only in some combined form such as

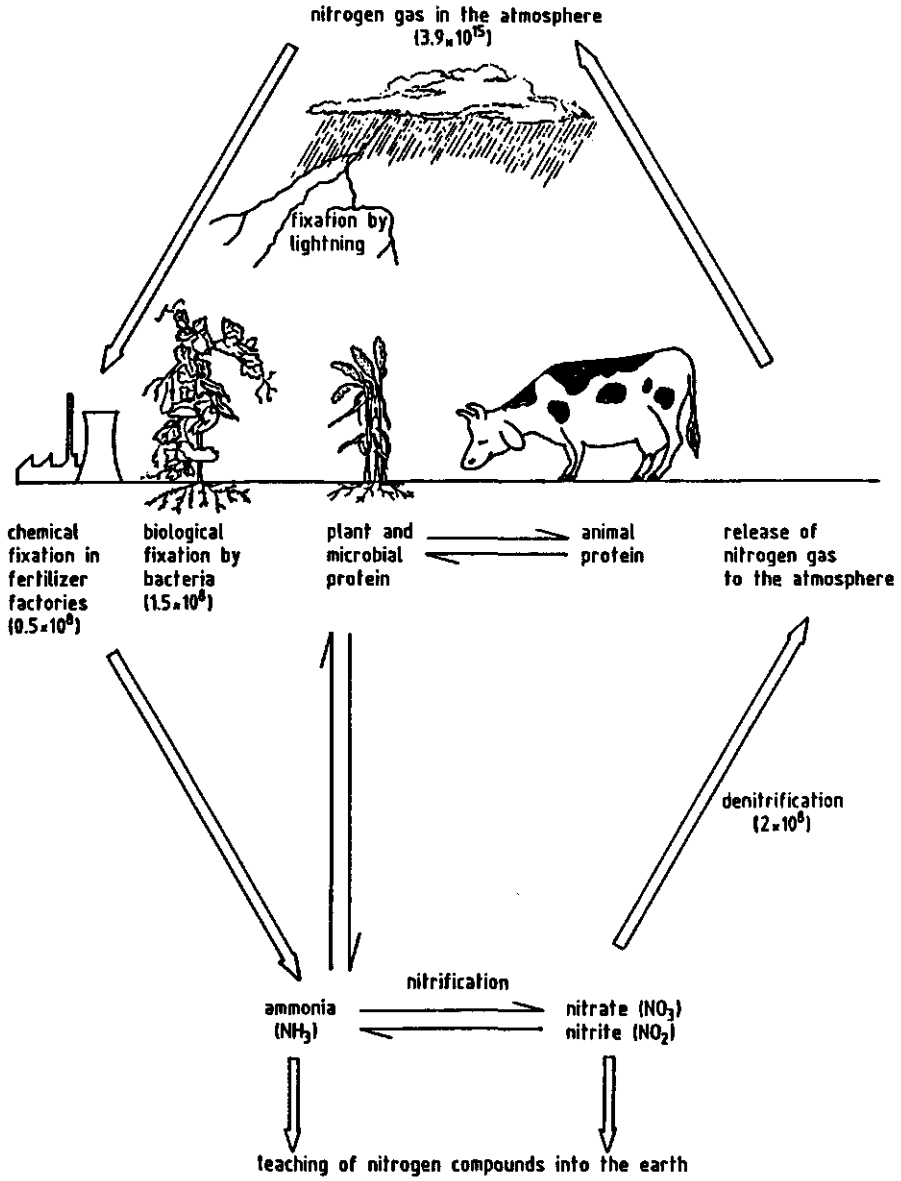


Fig. 1. **The global nitrogen cycle**. This simple schematic presentation of the nitrogen cycle shows the movement of nitrogen between plants, animals, microbes, the land and the atmosphere. The values between brackets are estimated turnover rates of nitrogen (tonnes/year).

nitrate, ammonia, or more complex compounds such as amino acids, that most organisms can utilise nitrogen. Green plants grow satisfactorily if their roots are supplied with nitrates or ammonium salts, and under natural conditions they can usually obtain these from the soil, or from the water if they are aquatics.

These simple nitrogen containing compounds are converted into proteins, nucleic acids and other nitrogenous components of the cell, a process called assimilation of nitrogen. Animals do not have such synthetic pathways and they acquire their nitrogen mainly by consuming, in their food, protein which has originally been synthesized by a plant. There is a continuous return of nitrogenous compounds, still in reduced form, to the soil as a result of decay and putrefaction of plant and animal material (ammonification). Soil microorganisms in turn can reoxidize NH_3 to form nitrite and nitrate (nitrification), which can then be utilized again by plants.

Thus nitrogen is constantly cycling, in a combined form, between the soil and the living organisms. But losses usually occur during this cycling. The loss of soil nitrate as N_2 and N_2O gases when denitrifying bacteria use it as oxidant anaerobically are estimated to be roughly 2×10^8 tonnes N per year (denitrification). Nitrogen fixation is the link in the nitrogen cycle that compensates for this net loss of nitrogen to the atmosphere.

1.2 Nitrogen Fixation

Next to water, the reduced form of nitrogen is the most frequently encountered limiting component for biological productivity on most areas of this planet. In agricultural areas, where a part of the plant growth is removed as a crop, the depletion of soil nitrogen is much more rapid. In modern, intensive agriculture, high yields are sustained only with synthetic ammonia-based or ammonia-derived fertilizers.

The synthesis of ammonia from dinitrogen and dihydrogen according to the Haber-Bosch process is carried out at around 350°C and 350-1000 Atm pressure on a cheap iron catalyst. The industrial fixation of nitrogen on a commercial scale requires factories which are able to produce large quantitaties of ammonia. This means that besides the energy costs to produce dihydrogen, there are the costs of storage and distribution. Although industrial nitrogen fixation has been a positive step towards better crops and the relief of hunger, such fixation involves a considerable usage of

limited and costly sources of energy. For the fixation of unit weight of nitrogen, the energy yielded by nearly twice that weight of oil is required. Thus, including the costs of storage and transport, fertilizer is rather expensive, perhaps too expensive for developing countries. However, most countries now have nitrogen fixing factories and the output of this industry has risen over the years. According to FAO statistics total world production in 1974 was 41 millions of tonnes of nitrogen. However, to compensate for the global loss of fixed nitrogen by denitrification, an annual input of some 2 x 10^8 tonnes of N must take place [1]. The bulk of this is produced by biological nitrogen fixation. Thus, even in highly developed agricultural countries, biological nitrogen fixation is the primary source of N-input into plant crops.

In order to meet the food expectations of the expanding population, an increase in agricultural productivity is required. Nitrogen is one of the principal limitations to increase world food production, so the need to bring more nitrogen into the food chain is evident. It has been suggested [3,4] that cereal grain production will have to double from 1.3 to 2.6 billion tonnes per year during the fourth quarter of this century. This means that the production of nitrogen fertilizer has to increase even more, because the average efficiency of use of fertilizer by crops is only about 50% [5]. However, both in developed and developing countries there is uncertainty about the cost and availibity of nitrogenous fertilizers, since the resources and the energy price for present methods of producing fertilizers are not inexhaustible and not predictable.
In addition, there is increasing concern over possible contribution of nitrogen fertilizers to water pollution. Therefore, in the long run it will be more sensible to make maximum use of biological nitrogen fixation, in order to avoid the depletion of fossil fuels and the damage to the environment that results from the liberal application of nitrogen fertilizers.

1.3 Biological Nitrogen Fixation

Only a few procaryotic microorganisms, the diazotrophs, have the capacity to convert dinitrogen from the atmosphere into NH_3, ammonia. This process called biological nitrogen fixation, makes use of very specialized biochemical systems, in which the enzyme nitrogenase catalyses the conversion of N_2 to NH_3. The synthesized NH_3 is used to build up proteins and other nitrogenous constituents of the cell. The inclusion of aerobic,

facultative, anaerobic and photosynthetic organisms among the diazotrophs attests to their physiological and metabolic diversity.

Nitrogen-fixing organisms are present in the free-living state in soils and in natural waters. When their cells die and decay, the newly-fixed nitrogen enters into general circulation. Free-living nitrogen-fixing microbes are rarely of serious importance in the terrestrial nitrogen economy. The only free-living organisms of serious agronomic importance are the blue-green algae which, being capable of photosynthesis, are not limited by availability of carbon substrates in soil. The most important contributors to the nitrogen cycle, from both ecological and agricultural points of view, are those diazotrophs that exist in association with higher plants, usually with its roots. These associations are generally called symbiosis. There are several forms of symbiosis described [6]. The most studied system is the legumes symbiosis of peas, beans, soybeans, lupins, lucerne and so on. The associations formed by leguminous plants with a species of the bacterial genus Rhizobium are the best known. After infection by Rhizobia little excrescences called nodules arise in the cortex of legume roots. The root nodule bacteria, called bacteroids, derive carbohydrates formed in photosynthesis from the host plant and use these in their growth, maintenance and nitrogen fixation. In return for carbohydrate supply, the higher plant receives for its own use the products of fixation. Thus, biological fixation of nitrogen is driven by solar energy, and proceeds at normal temperatures. Further it should be noted that between the extremes of a simple free-living nitrogen-fixing bacterium, such as Clostridium pasteurianum, and the highly-developed legume symbiosis, there is a very wide range of interorganismal relationships of varying complexity.

While about 30 years ago, biological nitrogen fixation was considered to be something associated with relatively primitive, low-input agriculture, current trends of agricultural production are towards increased utilization of legumes. By planting more hectares to legumes and by using good bacterial strains, in combination with improved plant cultivars and agronomic practices, biological nitrogen fixation can be increased. In addition, increased utilization of leguminous trees for timber and industrial uses is advocated.

1.4. Nitrogenase

1.4.1. Evolution

Three to four billion years ago the primordial earth was rich in compounds such as methane, ammonia, water, and to a lesser extent H_2, N_2, H_2S, CO and CO_2 [7,8]. The earliest successful life forms were unquestionably simple anaerobic organisms. The widespread ability of organisms to utilize ammonia as a source of nitrogen reflects the evolutionary significance of this nitrogen compound. With ammonia available the capacity to utilize N_2 would confer no selective advantage, and it is unlikely that nitrogenase developed so long as ammonia was plentiful. However, it is also speculated [9] that the enzyme nitrogenase originally discharged some different function, namely the reduction and detoxification of highly reactive compounds containing triple bonds to ensure the survival of primeval organisms. For, compounds present in the primordial environment such as cyanide, cyanogen, nitriles and isonitrile are known substrates of nitrogenase. Whether nitrogenase developed in response to a scarcity of combined nitrogen on earth at some stage in evolution, or as a detoxifying mechanism, the similarity of the nitrogenases among the diazotrophs suggests a common ancestry. But if the enzyme is so old, one may wonder why there has been so little evolutionary divergence in its structure. A reason for this might be the oxygen sensitivity of the nitrogenase proteins, which means that biological nitrogen fixation requires anaerobic conditions. During the development of higher organisms the property to fix nitrogen might have been lost as a result of the inability to perfect a mechanism to allow nitrogen fixation in an aerobic environment. For aerobically nitrogen fixing bacteria such as Azotobacter vinelandii several protection mechanisms against oxygen have been described [10,11].

1.4.2. Molecular properties

The nitrogenase enzyme complex consists of two proteins, both of which are required for catalytic activity. The proteins are readily separated and purified independently. The larger and more complex component of nitrogenase, the MoFe protein, is an $\alpha_2\beta_2$ tetramer with a molar mass near 220 kDa [12]. The molar masses for the two subunits are approximately 50 and 60 kDa [13]. The other protein component of nitrogenase, the Fe protein, is a dimer composed of two identical subunits and has a reported molar mass bet-

ween 55 and 70 kDa, depending upon the source of the protein [14-17]. There seem to be only slight physical differences in the proteins as isolated from a variety of different nitrogen fixing species. The amino acid sequence of the MoFe- and Fe proteins of several species has been determined, mostly from DNA sequencing of the corresponding nif genes [18-22]. There is a high degree of homology, the major divergence has been observed with C.pasteurianum protein components.

Although the component proteins from a number of organisms have been purified to give homogeneous preparations when examined by SDS polyacrylamide gel electrophoresis, homogeneity with respect to the catalytic center(s) still remains problematic. The specific activities of the component proteins used in different studies vary considerably. Part of this variation may be due to species differences since not all nitrogenases ought to have the same turnover number. Compare for example the specific activities of the nitrogenases of R.japonicum and K.pneumoniae with those of A.vinelandii and C.pasteurianum: 1000 and 600 for Rj_1 [23] and Rj_2 [23], 2150 and 1600 for Kp_1 [24] and Kp_2 [25], 3600 and 3400 for Av_1 [26] and Av_2 [27], 2500 and 3100 for Cp_1 [28] and Cp_2 [28]. So it is not clear what the maximal specific activity is of each of these nitrogenase proteins. Some authors correct their experimental results for the presence of inactive protein. The concentration of active MoFe protein in a preparation is mostly related to its Mo content, taking two Mo atoms per molecule of MoFe protein as 100% active [29,30]. Lowe and Thorneley [29] calculated the percentage of inactive Kp_2 in their preparation by assuming a maximum specific activity of 3500. Hageman et al. [30] used the reaction of Fe protein with BPS in the presence of MgATP [31] to quantitate the amount of Fe_4S_4 cluster per molecule of Av_2, taking one Fe_4S_4 cluster per Av_2 as 100% active Fe protein.

Knowledge of the metal and acid-labile sulfur (S^{2-}) composition of 100% active MoFe protein and Fe protein is necessary for identification of the different clusters present in the nitrogenase proteins. Metal analyses of the MoFe protein from different species indicate 2 Mo atoms, 22 to 34 Fe atoms, and an amount of acid-labile sulfur roughly comparable to that of Fe [32-34]. The Mo atoms and some of the Fe and S^{2-} atoms are contained in an extractable cofactor, called FeMoco [35] that is responsible for the characteristic EPR spectrum of the MoFe protein, with g values near 4.3, 3.7 and 2.01 [32,36]. Recently, the excistance of an alternative nitrogenase system has been proven. In this system vanadium instead of molybdenum

is used as the substrate binding site [37]. It is biochemically a different enzyme since the polypetides are coded by different genes. But the basic build-up of the vanadium nitrogenase is remarkable similar to the Mo system.

It was generally accepted that Fe protein contains 4 Fe atoms and 4 labile S atoms, arranged in a cubane like Fe_4S_4 cluster [33]. However, work of Braaksma et al. [38] and Haaker et al. [39] showed that, depending on the growth conditions of the cells, Fe protein with more than 4 Fe atoms can be isolated. The dithionite-reduced Fe protein of all nitrogenases has an EPR signal of the "1.94 type", with a spin type of S=½. Double integration of the EPR spectrum gives values ranging from 0.2 to 0.5 electrons per dimeric protein molecule [32,33,40]. Recently it has been shown that Fe protein from A.vinelandii, A. chroococcum and K.pneumoniae also exhibits a weak EPR signal with g ≈ 5 [41,42]. Upon integration it was found that this cluster accommodates 0.5 - 0.8 electrons with a spin state of S=3/2.

Most of the variations in specific activity and metal content of different preparations of MoFe protein and Fe protein have been explained by the extreme oxygen sensitivity of the nitrogenase proteins. Since important differences in experimental results might appear when highly active proteins are used instead of low active preparations, conclusions drawn from studies with the nitrogenase proteins should always be judged in the light of possible active-site heterogeneity.

1.4.3. Mechanism of nitrogenase catalysis

Nitrogenase is capable of reducing a large number of double- and triply-bonded molecules [43,44]. During the reduction of the natural substrate N_2, at least 25% of the total flux of electrons through nitrogenase is allocated to H^+ [45]:

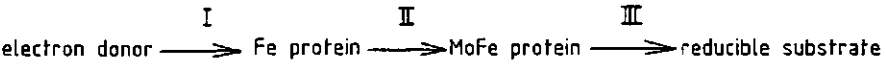
$$N_2 + 8\ H^+ + 8e \longrightarrow 2\ NH_3 + H_2$$

Although in the absence of reducible substrates such as N_2, electron consumption by nitrogenase is not inhibited and protons are reduced to H_2, there is evidence that H_2 evolution is an integral part of the enzymic reduction of N_2. It is suggested [46,47] that N_2 binds to the MoFe cofactor and replaces bound H_2.
Besides a source of low-potential reducing equivalents and the absence of

oxygen, MgATP is required in the nitrogenase reactions. The hydrolysis of at least 16 molecules of MgATP is necessary for the reduction of one molecule of N_2 [48,49]. Under less optimal conditions for substrate reduction, uncoupling occurs between the electron consumption of nitrogenase and its ATPase activity. Moreover, in the absence of low-potential reducing equivalents nitrogenase shows a low rate of MgATP hydrolysis, called the reductant-independent ATPase activity [50-54].

The direction of electron flow through nitrogenase was established primarily on the basis of experiments with EPR spectroscopy [55,56]:

Scheme I



1.4.3.1. Reduction of Fe protein (I).

The kinetics of the reduction of oxidized Fe protein with $Na_2S_2O_4$ have been investigated with stopped-flow spectrophotometry and rapid-freezing EPR spectroscopy [57,58]. A second-order rate constant for the reduction of oxidized Fe protein by $SO_2^{\bullet-}$ of $k > 10^8\ M^{-1}s^{-1}$ has been estimated [58]. Kinetic data of the reduction of oxidized Fe protein under different experimental conditions give important information about the reaction mechanism of nitrogenase catalysis, since after electron transfer from Fe protein to MoFe protein oxidized Fe protein will be associated to MoFe protein with MgATP, MgADP and/or P_i bound. Stopped-flow experiments in which oxidized Fe protein was mixed with a solution of $Na_2S_2O_4$ plus either MgATP or MgADP showed that MgATP did not influence the rate of reduction of Fe protein, while MgADP decreased the rate constant to a value of $3 \times 10^{-6}\ M^{-1}s^{-1}$ [25]. The effect of MgADP on the rate of reduction means that MgADP binds to oxidized Fe protein in less than 1 ms, thereby inhibiting the electron transfer from $SO_2^{\bullet-}$ to Fe protein. From these experiments it is not clear whether the inability of MgATP to effect the rate of reduction of oxidized Fe protein is due to a slower rate of association of MgATP to oxidized Fe protein, or that binding of MgATP to oxidized Fe protein has no effect on the rate of reduction by $SO_2^{\bullet-}$. The rate of reduction of oxidized Fe protein by $SO_2^{\bullet-}$ in the presence of MgADP is strongly inhibited by the binding of

Fe protein to the MoFe protein [25]. These effects have been explained by assuming that the complex of oxidized Fe protein with MoFe protein and MgADP is either reduced very slowly or not at all by $SO_2^{\bullet -}$ [25].

The redox properties of the Fe/S clusters present in Fe protein were studied by EPR, absorbance and CD spectroscopy and microcoulometry. Watt [59] showed that the S=½ EPR signal of oxidized Av_2 could be fully developed by the addition of one electron equivalent. It was also shown with microcoulometric reduction that the midpoint potential of Av_2 in the absence of adenine nucleotides is -310 mV and in the presence of MgATP and MgADP the midpoint potential shifts to -425 mV and -475 mV respectively. Braaksma et al. [60] reported a value of -393 mV but this value might not be reliable since high concentrations of Na_2SO_3 are necessary to obtain potentials above -360 mV and these concentrations might interfere with the EPR quantitation.

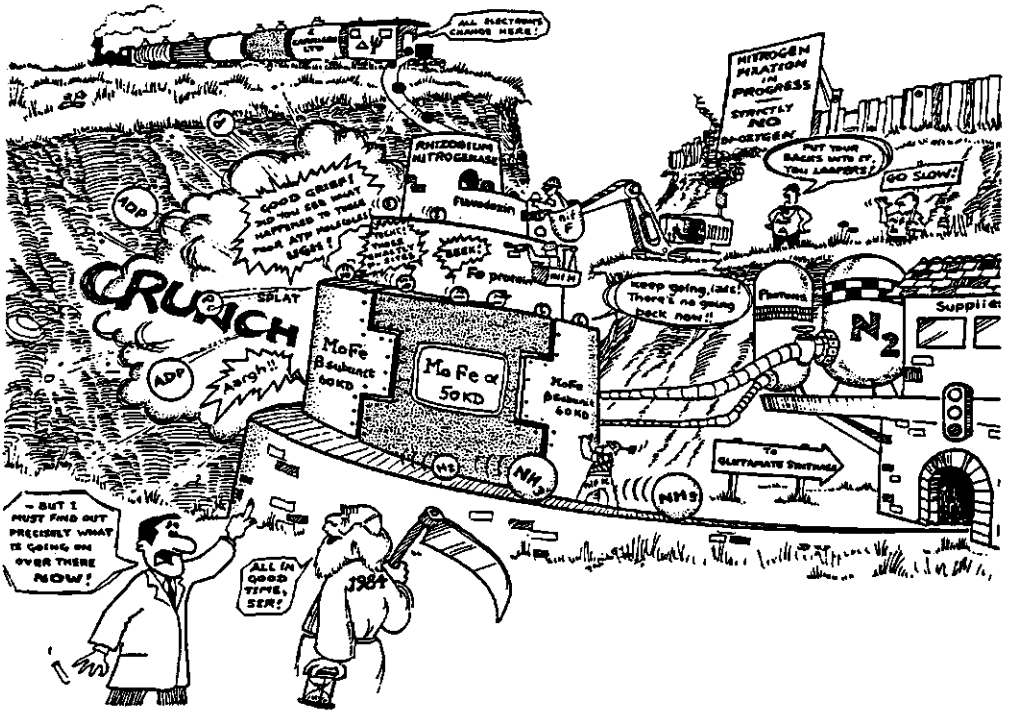


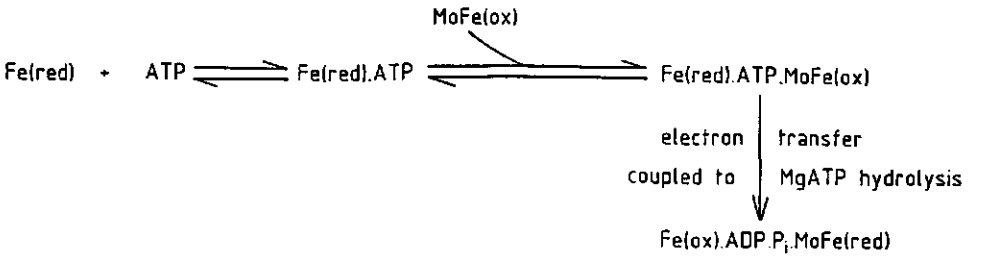
Fig. 2. This drawing showing the mechanism of nitrogenase action was reproduced, with permission, from ref. [88].

Recently, Morgan et al. [61] showed by monitoring the intensity of the EPR signal of the cluster with spin state S=1/2 and S=3/2 that both clusters have the same redox potential. This observation explains why Watt [59] found one electron equivalent associated with the total redox changes in Av_2 while monitoring the S=1/2 signal which accounts only for 20% of the clusters present in Fe protein. It is not clear yet whether both clusters can act as efficient electron donors for MoFe protein.
Little information is available about the actual redox potential necessary for N_2 fixation, but Braaksma et al. [60] showed for nitrogenase of A.vinelandii that the redox potential required for half-maximum rates of H_2 production is approximately -390 mV. It is not known whether there is an obligatory order for the Fe protein to bind MgATP and be reduced by dithionite or whether these processes occur at random. Although studied extensively [62-65], it is not yet clear whether Fe protein has to be considered as a one- or as a two-electron acceptor/donor during nitrogenase turnover.

1.4.3.2. Electron transfer from Fe protein to MoFe protein (II).

The electron transfer reaction from Fe protein to MoFe protein was studied by stopped-flow spectrophotometry. Since oxidation of the Fe protein is associated with an increase in absorption at 425 nm, the time course of the electron transfer reaction can be followed in a stopped-flow apparatus. There is only electron transfer in the presence of MgATP. The rate of electron transfer is independent of the order of mixing of Fe protein, MoFe protein, and MgATP [66], and this supports a random mechanism of the first two steps in Scheme II:

Scheme II



Furthermore, the rate of electron transfer is dependent upon the MgATP concentration and independent of the concentration of Fe protein and MoFe protein [30,67]. This indicates that the formation of a ternary complex between Fe protein, MoFe protein and MgATP is relatively fast compared to the rate of electron transfer. The rate of association of Fe(red).ATP with MoFe(ox) has been estimated to be larger than 10^7 $M^{-1}s^{-1}$, with a dissociation constant in the order of 0.5 μM [68]. The stoichiometry of the active complex is still uncertain, since ratios of Fe protein to MoFe protein of both 2 to 1 and 1 to 1 have been reported as optimal for nitrogenase activity [27,69-72].

Since binding experiments of MgATP to the complex of Fe protein and MoFe protein are not possible due to the hydrolysis of MgATP by the complex, only binding data are available of MgATP to Fe protein alone. Fe protein probably has two binding sites for MgATP [31,73-75].

At saturating MgATP concentrations, a rate constant of 200 s^{-1} was determined for the electron transfer between the nitrogenase proteins of K.pneumoniae at 23°C, which is much faster than the turnover time of nitrogenase at that temperature (~ 6.4 s^{-1}).

1.4.3.3. Substrate reduction (III)

The electrons donated by the Fe protein to the MoFe protein are transferred within the MoFe protein to the various FeS centers and the FeMoco center. There is now direct evidence available that the locus of substrate reduction is on the MoFe protein [76]. Studies with acetylene and N_2 demonstrate that FeMoco is the site of substrate binding and reduction [77-79].

When it is assumed that Fe protein acts as a one electron donor/acceptor, then the electron transfers from Fe protein to MoFe protein must proceed in single electron steps. Thus for a 1:2 complex of MoFe protein and Fe protein maximally two electrons can be transferred simultaneously from Fe protein to MoFe protein. Since MoFe protein has two FeMoco clusters and thus presumably two catalytic sites, only one electron per catalytic site is transferred. This means that even in the case of H_2 production by nitrogenase, the reaction pathway shown in scheme II must be completed at least two times before the substrate on the MoFe protein is fully reduced. It has been suggested that oxidized Fe protein must dissociate from MoFe protein

before re-reduction by dithionite can occur [25]. Thus the two proteins must dissociate after each electron is transferred from the Fe protein to the MoFe protein. Thorneley and Lowe [25] demonstrated that the rate of dissociation of oxidized Fe protein from MoFe protein is in the same order of magnitude as the turnover time of nitrogenase.

1.4.4. The role of MgATP in nitrogenase catalysis.

One of the most intriguing and perhaps least resolved problems of the nitrogenase reaction is the mechanism of ATP action. Unfortunately, no model is available. The overall chemical reduction of N_2 to NH_3 by H_2 is a thermodynamically favourable reaction [80]. The energy liberated by MgATP hydrolysis is therefore not needed to drive an otherwise unfavourable equilibrium in the desired direction. However, the reaction pathways for the formation of the possible intermediates N_2H_2 and N_2H_4 are both endergonic (see scheme III).

Scheme III

$N_2 + 2H^+ + 2\ e \leftrightarrows N_2H_2 \qquad E^{\circ 1} = -1450$ mV

$N_2 + 4H^+ + 4\ e \leftrightarrows N_2H_4 \qquad E^{\circ 1} = -695$ mV

$N_2 + 6H^+ + 6\ e \leftrightarrows 2NH_3 \qquad E^{\circ 1} = -280$ mV

$2H^+ + 2\ e \leftrightarrows H_2 \qquad E^{\circ 1} = -420$ mV

Thus the ATP requirement can be understood in light of the low potential electron donor needed for the reduction of the stable N≡N bond to form N_2H_2 and N_2H_4. It is questionable whether N_2H_2 is an intermediate, but N_2H_4 has been found as such [81].

Besides the reduction of the physiological substrate N_2, all other reductions catalyzed by nitrogenase need the hydrolysis of MgATP. The minimum amount of MgATP hydrolyzed seems to be two molecules of MgATP per electron transferred to substrate (ATP/e $\geq$ 2) [82]. There seems to be no upper limit to the ATP/e ratio, since MgATP hydrolysis can be totally uncoupled from electron transfer (reductant-independent ATPase activity). The ATP/e ratio can be varied by varying the temperature [49,51], the pH [54], the ratio of the component proteins of nitrogenase [69], the reductant concentration [27], and also by using heterologous combinations of component proteins [83,84].

The minimum value of 2 MgATP's hydrolyzed per electron transferred fits

nicely in a model derived from experiments indicating two binding sites for MgATP on the Fe protein and the transfer of one electron from the Fe_4S_4 cluster on the Fe protein onto the MoFe protein [64]. Furthermore, Eady et al. [85] have shown by a rapid-quench experiment that MgATP is hydrolyzed at a rate equal to the rate of initial electron transfer between the nitrogenase proteins of K.pneumoniae at 10°C. Although the time course of MgATP hydrolysis was indistinguisable from that of electron transfer, the stoichiometry of the hydrolytic reaction remained uncertain. From analysis of the pre-steady-state burst of MgATP hydrolysis by nitrogenase of A.vinelandii at 30°C Hageman et al. [30] concluded that 2.5 molecules of MgATP are hydrolyzed per electron transferred from the Fe_4S_4 cluster of the Fe protein. They suggest that all of the known effects of MgATP in the overall nitrogenase reaction can be accounted for by its function in the electron transfer between the two proteins of nitrogenase. However, additional roles for MgATP in nitrogenase catalysis have been proposed by Eady et al. [86]. They suggested that during turnover an additional binding site for MgATP on the MoFe protein is generated, with a role in substrate reduction.
Stiefel [87] suggested a direct interaction between MgATP and the substrate reduction site, mainly on the basis of model chemistry.

1.5. Comparison of Nitrogenase with other ATPases

Several distinct biological systems are known in nature which use the energy of ATP hydrolysis to drive energy-consuming processes, such as the synthesis of biomolecules, active transport and mechanical work in muscle tissue. The problem of devising a mechanism for ATP-linked transport is fundamentally different from the problem of devising a mechanism for catalysis of chemical transformations. In a chemical transformation, the enzyme functions as a rate accelerator of a chemical reaction that in principle could occur in the absence of an enzyme. During active transport there is exchange of free energy, but not of matter, between the transported species and ATP. During nitrogenase catalysis the energy of ATP hydrolysis is used to transfer an electron from the Fe protein to the MoFe protein. At present it is not known how the chemical energy of ATP is utilized in redox reactions.
The possibility that there is a general principle for free energy coupling in the processes of electron transfer (nitrogenase) and cation transfer (transport proteins) will be considered. Therefore in this section a concise discussion will be given of some of the mechanisms proposed for free energy transfer in

active transport systems. These ideas might be helpful in the formulation of a molecular mechanism for the nitrogenase reaction.

Two different types of transport proteins are now known to exist. Although both types of proteins are membrane-bound ATPases, there is a great difference in their molecular composition and also their physiological function. One class of ATPases includes the (Na^+,K^+)-ATPase of animal cells [89,90], Ca^{2+}-ATPase of sarcoplasmic reticulum [91] or erythrocyte plasma membrane, (H^+,K^+)-ATPase of gastric mucosa [92] and the H^+-ATPase of fungi [93]. Each of these enzymes has a very simple structure, with one major subunit of molar mass of about 100 kDa. The enzymes appear to function physiologically either directly or indirectly in transport processes. In the (Na^+,K^+)- and Ca^{2+}-pumps and in the gastric and fungal H^+-pumps, ATP processing occurs via an acyl phosphate intermediate, in which the phosphate is covalently linked to an aspartyl residue of the protein [92].

The other type of ATPases, the F_0F_1 class, is also scattered widely throughout the biological world, and is found in bacteria [94,95], mitochondria [96,97], chloroplasts [98], chromaffin granules and the envelopes of certain animal viruses. The physiological function of the H^+-ATPases is the catalysis of ATP synthesis in a process called oxidative phosphorylation (photophosphorylation in chloroplasts and photosynthetic bacteria). In bacteria, mitochondria and chloroplasts, during oxidative- or photophosphorylation, H^+ is driven across the membrane during electron transport through an electron transport chain and returned through the F_0F_1 complex to synthesize ATP (chemiosmotic hypothesis of Mitchell [99]). In the absence of redox reactions, on the other hand, the H^+-ATPase could drive H^+ in the reverse direction. The subunit composition of H^+-ATPases is highly complex. The enzyme consists of two structurally distinct components, a membrane-bound sector, F_0, which functions as a proton-translocating channel, and an extramembrane sector, F_1, which has catalytic activity (ATPase turnover 600 s^{-1}). F_0 can be extracted from the membrane by the use of detergents and contains three or four distinct proteins [100-102]. F_1 can be detached from the membrane as a water soluble complex of five distinct subunits, α to ϵ [103,104]. In the last few years strong evidence has accumulated suggesting that the subunit stoichiometry is actually $\alpha_3\beta_3\gamma\delta\epsilon$. F_1 contains both catalytic and regulatory sites that bind adenine nucleotides [105]. Although the total number of these sites is not firmly established, it appears that up to six adenine nucleotide binding sites are present on F_1 [106,107]. Three of these binding sites are normally occupied by firmly bound, non-exchangeable ATP or ADP, and these sites presumably have a structural role.

The catalytic site(s) is most likely located on the β subunit. Binding of phosphate to F_1 has also been demonstrated [108].

1.5.1. **Models for ATP synthesis**

The chemiosmotic hypothesis of Mitchell [99] gives a general idea of how ATP synthesis in oxidative phosphorylation is supplied with energy. Mitchell proposes that electron transport in the respiratory chain driven by a difference in redox potential of redox couples, gives rise via the translocation of protons across the membrane, to an electrochemical potential gradient for protons (proton-motive force). The proton motive force $\Delta\mu_{H^+}$ may be expressed as the sum of a proton concentration gradient (ΔpH) and a membrane potential ($\Delta\psi$). The proton-motive force is a storage form of energy utilizable by the H^+-ATPase for ATP synthesis. Although the principles of Mitchell's chemiosmotic hypothesis has been widely accepted, the major questions that remain unresolved deal with attempts to obtain a chemical description of how the gradient is formed and how the flow of protons down their electrochemical gradient and through the F_OF_1 enzyme is linked to the synthesis of ATP. For instance a detailed understanding of the mechanism of proton translocation and identification of the specific hydrogen carrier in each segment of the respiratory chain has not yet been achieved, but it is generally recognized that the components of the respiratory chain are involved in proton translocations [109].
The second and equally challenging problem is how the proton flux is utilized to generate ATP from ADP and P_i. Hypotheses in which the proton-motive force is proposed to be directly involved in ATP generation are referred to as direct mechanisms. When the driving force for ATP synthesis is a conformational change of the ATPase induced by protons the mechanism is called indirect.

1.5.1.1. Direct Mechanisms

Before 1973, all popular models for oxidative phosphorylation and photophosphorylation assumed that the energy, in whatever form it was transferred, would be directly used to form the ADP-P_i anhydride bond. According to Mitchell's formulation [110], P_i is directly accessible to the proton flux at the active site of the ATPase ("ligand conduction" mechanism). Specific protonation of P_i is accompanied by a concerted attack by ADP resulting in the formation and subsequent release of ATP. However, the experimental evidence for such a confluent pathway is not impressive.

STELLINGEN

1. De experimentele resultaten van Reddy en Vaidyanathan rechtvaardigen niet de konklusie dat benzoate-4-hydroxylase tetrahydropteridine als cofaktor nodig heeft voor enzymatische aktiviteit.

   Reddy, C.C. & Vaidyanathan, C.S. (1975) Biochim. Biophys. Acta 348, 46-57.

2. Uit de door Tso en Burris beschreven bindingsexperimenten van ATP en ADP aan komponent 2 van nitrogenase uit Clostridium pasteurianum valt niet op te maken in welke oxidatietoestand het nitrogenase eiwit zich bevindt tijdens de gebruikte gel equilibratie methode.

   Tso, M-Y. & Burris, R.H. (1973) Biochim. Biophys. Acta 309, 263-270.

3. De konklusies die Hageman et al. trekken uit één enkel experiment, waarbij de hoeveelheid fosfaat, die vrijkomt tijdens de pre-steady-state hydrolyse van ATP door nitrogenase van Azotobacter vinelandii, bepaald werd, getuigen van "wishful thinking" en blijken bovendien onjuist te zijn.

   Hageman, R.V., Orme-Johnson, W.H. & Burris, R.H. (1980) Biochemistry 19, 2333-2342.
   Dit proefschrift.

4. Aktiviteitsmetingen van pteridine-afhankelijke mono-oxygenasen door bepaling van de oxydatiesnelheid van NAD(P)H in een assay waaraan een pteridine verbinding als cofaktor is toegevoegd zijn onbetrouwbaar.

   Shailubhai, K., Sahasrabudhe, S.R., Vora, K.A. & Modi, V.V. (1983) FEMS Microbiol. Lett. 18, 279-282.
   Kaufman, S. (1979) J. Biol. Chem. 254, 5150-5154.

5. Studies naar induktie, repressie en inhibitie van amylase door o.a. zetmeel en glucose bij hyfenvormende schimmels als Neurospora crassa en enkele Aspergillus soorten leveren zoveel verschillende en tevens tegenstrijdige konklusies op, dat het aanbeveling verdient de verschillende aspekten in één en dezelfde studie te analyseren.

6. De bewering dat met het huidige energieverbruik op korte termijn een tekort aan fossiele energiebronnen zal ontstaan, is gebaseerd op verkeerde schattingen van deze brandstofvoorraden en wordt dan ook ten onrechte als argument aangevoerd voor de noodzaak van kernenergie.

7. Bindingsstudies waarbij het maximale aantal bindingsplaatsen van een ligand op een eiwit bepaald wordt door gebruik te maken van ligand geïnduceerde spektrale veranderingen, zoals circulair dichroisme of ESR, van het betreffende eiwit zijn diskutabel.

   McKenna, C.E., Stephens, D.J., Eran, H., Luo, G.M., Matai Ding, F.X.Z. & Nguyen, H.T. (1984) in Advances in nitrogen fixation research (Veeger, C. & Newton, W.E., eds.) pp. 115-122, Nijhoff/Junk, Pudoc.

   Zumft, W.G., Palmer, G. & Mortenson, L.E. (1973) Biochim. Biophys. Acta 292, 413-421.

8. Nadat de EEG jarenlang de landbouwproduktie door subsidies heeft gestimuleerd, is het niet rechtvaardig de inkomenseffekten als gevolg van gedwongen produktiebeperkingen alleen tot het bedrijfsrisiko van de agrariër te rekenen.

9. In de talloze handboeken over enzymkinetiek worden in het algemeen dezelfde soort reaktiemechanismen beschreven. Het uitwerken van een reaktiemechanisme voor een enzym dat uit meerdere dissocieerbare eiwitkomponenten bestaat die samen nodig zijn voor enzymaktiviteit zou in dit opzicht vernieuwend zijn.

10. Men kan zich afvragen of het invoeren van de APK-autokeuring niet meer bijdraagt tot het rendement van garages dan tot de verkeersveiligheid.

11. Het blijkt overduidelijk uit de kwaliteitsvergelijking van de fietspaden en van de autowegen, dat de Nederlander pas bepaalde voorrechten geniet als er belasting voor betaald is.

Jan Cordewener
Wageningen, 7 mei 1987

1.5.1.2. Indirect Mechanisms

(a) Phosphoenzyme intermediate hypothesis.

ATP processing in Ca^{2+}-and (Na^+,K^+)- ATPases occurs via an acylphosphate intermediate. This intermediate exists in two very different (conformational) states, a high free energy phosphoenzyme intermediate (E~P) and a low free energy phosphoenzyme intermediate (E'-P), of which the former can react with ADP to form ATP. One hypothesis proposes that the interaction of Mg^{2+} with the protein induces a conformational change resulting in phosphoenzyme formation. The function of the proton gradient is to displace the firmly bound Mg^{2+} from the enzyme [111]. However, all available evidence indicates that ATP processing by the F_OF_1 proteins does not involve phosphoenzyme intermediates. It has been shown that the bovine H^+-ATPase catalyzed hydrolysis of isotopically labeled ATPγS results in inversion at the γ phosphorus [112]. This suggests a one-step mechanism for ATP hydrolysis, in which the phosphoryl group is transferred directly from ATP to water without participation of a phosphorylated intermediate. The same has been shown for ATP hydrolysis by nitrogenase [113].

(b) Boyer-Slater hypothesis (energy-linked binding change mechanism).

In a direct mechanism the energy available from the electrochemical gradient is used to drive the synthesis of ATP at the catalytic site, namely by direct interaction of translocated H^+-ions with the chemical substrates. The idea that major energy transformations occur during binding changes rather than during synthesis of phosphoric acid anhydride bonds is based on several experimental observations, such as a differential sensitivity of the catalytic step to uncouplers and the finding of tightly bound nucleotides to F_1. To explain all their experimental data, Slater and coworkers [114,115] and Boyer and coworkers [116,117] proposed an energy-linked binding-change mechanism, also referred to as the alternating site model. From isotope exchange experiments it was concluded that the equilibrium constant for the ATP hydrolysis reaction is close to unity [118]. This means that the standard free energy of the reaction is approximately zero ($\Delta G^\circ \simeq 0$) when it occurs between enzyme-bound compounds. This leads to a need for tightly and loosely bound substrate species. Namely, when ATP is close to equilibrium with ADP and P_i at the catalytic site, a major energy transduction step will be the conversion of the ATPase enzyme into a conformational state that binds ATP or ADP/P_i weakly. However, proof in molecular terms for conformational events is difficult to obtain. Boyer *et al.* [119] have proposed a model in which the F_OF_1 complex is capable of coupling the energy available when protons move down an electrochemical gradient to an energy-

dependent conformational change in F_1 with a consequent change in the binding energies of substrates and products. Fig. 3 shows a schematic representation of such a model. The squares and circles represent different conformations of the subunits of the enzyme. In the square conformation enzyme-bound ADP, P_i and ATP are each in equilibrium with its counterpart species in the medium (adenine nucleotides are loosely bound), and in the circle conformation enzyme-bound ADP and P_i are in equilibrium with enzyme-bound ATP (adenine nucleotides are tightly bound). Several lines of evidence (isotope exchange experiments) indicate that ADP binding is necessary for ATP release during synthesis while the release of ADP in hydrolysis is enhanced by ATP binding. These observations of cooperative interaction between the catalytic subunits is expressed in the model of Fig. 3 by assuming that both subunits always possess different conformations (alternating site mechanism).

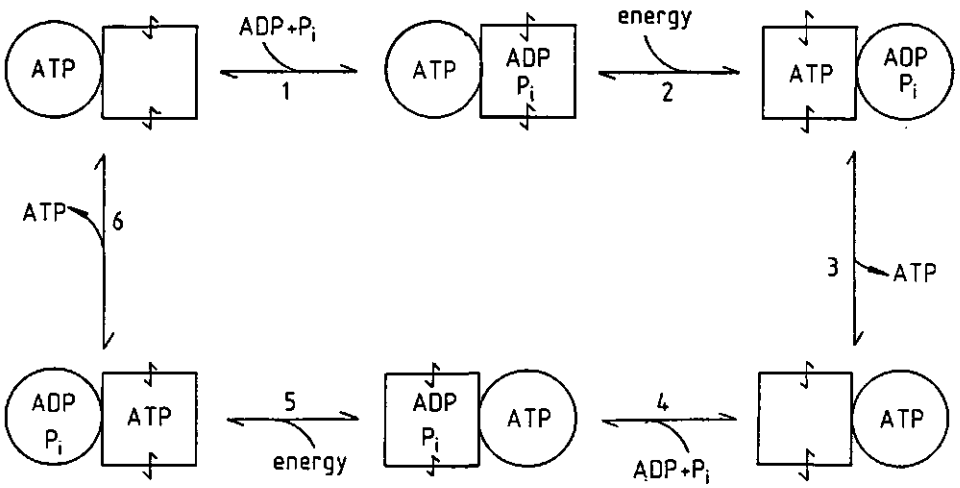


Fig. 3. A schematic representation of the energy-dependent binding change mechanism (from ref. 120, slightly modified).

For example, the enzyme might start out with ATP tightly bound to the circle conformation. In step 1, ADP and P_i are bound to the square conformation. In step 2 energy from proton translocation is used to switch the conformation of the two subunits, resulting in the conversion of tightly bound ATP to loosely bound ATP and simultaneously of the conversion of loosely bound ADP and P_i to tightly bound ADP and P_i. In step 3 there is release of ATP and formation of bound ATP from bound ADP and P_i at the catalytic site with no additional energy input. This tightly bound ATP in turn is not released until ADP and P_i bind at a

second site (step 4). The model in Fig. 3 does not take into consideration how proton translocation might be coupled to protein conformational change. It was proposed that in response to electrochemical proton gradients conformational changes in F_0 occur as a result of changes in ionization of charged amino acid residues of the subunit proteins. These changes in F_0, transmitted to F_1 could bring about changes in the conformation of F_1. The resulting reduced affinity of the catalytic sites for ATP was considered to arise from these changes in F_1.

1.6 Outline of this thesis

At the time this research was started little information was available about the role of MgATP in nitrogenase catalysis. Some investigators proposed that the only role of MgATP is in support of the electron transfer from Fe protein to MoFe protein, while others suggested the existence of a second role for MgATP. The aim of the present study was to get more insight in the mechanism of action of MgATP in nitrogenase catalysis.

Chapters 2 and 3 describe the binding properties of the nitrogenase proteins from A.vinelandii for MgATP and MgADP. The binding of these adenine nucleotides, separately and in competition experiments, was investigated to both oxidation states of Av_2. Also the binding of MgADP to the complex of Av_1 and Av_2 was studied. Chapter 4 deals with pre-steady-state measurements performed to quantitate the amount of MgATP hydroylzed by nitrogenase during the burst reaction under different experimental conditions. From these data it is concluded that the present model for MgATP hydrolysis by nitrogenase is too simple, particularly with respect to the function of MgATP in electron transfer. Chapter 5 presents steady-state data of the ATPase activity of nitrogenase, both in the presence and in the absence of reductant. The kinetic properties of the reductant-dependent and reductant-independent ATPase activity are explained in terms of a model that predicts the existence of a relatively stable intermediate of Av_2.
The results presented in this thesis will be discussed in Chapter 6, which is followed by a summary in Dutch.

References

1. Burns, R.C. & Hardy, R.W.F (1975) Nitrogen Fixation in Bacteria and Higher Plants, pp. 43-60, Springer-Verlag, Berlin, Heidelberg, New York.
2. Burrio, R.H.(1977) In: Genetic Engineering for Nitrogen Fixation (Hollaender, A., ed.) pp. 9-18, Plenum Press, New York, London.
3. Hardy, R.W.F. (1977) In: Report of the Public Meetings on Genetic Engineering for Nitrogen Fixation, pp. 77-106, U.S. Government Printing
4. Hardy, R.W.F., Havelka, U.D. & Quebedeaux, B. (1978) In: Proc.Natl.Int. Congress on Photosynthesis (Hall, D.O., Coombs, J. & Goodwin, T.W. eds.) pp. 695-719, The Bioch.Society, London.
5. Huber, D.M., Warren, H.L., Nelson, D.W. & Tsai, C.Y. (1977) Bioscience 27, 523-529.
6. Quispel, A. (1974) In: The biology of nitrogen fixation (Quispel, A., ed.) pp. 1-8, North-Holland Publishing Company, Amsterdam, The Netherlands.
7. Oparin, A.I. (1964) Life: Its Origin, Nature and Development, Academic Press, New York.
8. Beckner, L.V. & Marshall, L.C. (1965) Proc.Natl.Acad.Sci. USA 53, 1215-1225.
9. Silver, W.S. & Postgate, J.R. (1973) J.theoret.Biol. 40, 1
10. Robson, R. & Postgate, J.R. (1980) Ann.Rev.Microbiol. 34, 183-207.
11. Gallon, J.R. (1981) Trends Biochem.Sci. 6, 19-23.
12. Lowe, D.J., Smith, B.E. & Eady, R.R. (1980) In: Recent Advances in Biological Nitrogen Fixation (Subba Rao, N.S., ed.) pp. 34-87, Edward Arnold, London.
13. Brigle, K.E., Newton, W.E. & Dean, D.R. (1985) Gene 37, 37-44.
14. Tanaka, M., Haniu, M. Yasunobu, K.T. & Mortenson, L.E. (1977) J.Biol.Chem. 252, 7081-7100.
15. Mevarech, M., Rice, D. & Haselkorn, R. (1980) Proc.Natl.Acad.Sci. USA 77, 6476-6480.
16. Sundaresan, V. & Ausubel, F.M. (1981) J.Biol.Chem. 256, 2808-2812.
17. Hausinger, R.R. & Howard, J.B. (1982) J.Biol.Chem. 257, 2483-2490.
18. Mazur, B.J. & Chiu, C-F. (1982) Proc.Natl.Acad.Sci. USA 79, 6782-6786.
19. Lammers, P.J. & Haselkorn, R. (1983) Proc.Natl.Acad.Sci. USA 80, 4723-4727.
20. Yun, A.C. & Szalay, A.A. (1984) Proc.Natl.Acad.Sci. USA 81, 7358-7362.
21. Hase, T., Wakabayashi, S., Nakamo, T., Zumft, W. & Matsubara, H. (1984) FEBS Lett. 166, 39-43.

22. Kaluza, K. & Hennecke, H. (1984) Mol.Gen.Genet. 196, 35-42.
23. Israel, D.W., Howard, R.L., Evans, H.J. & Russell, S.A. (1974) J.Biol.Chem. 249, 500-508.
24. Smith, B.E., Thorneley, R.N.F., Yates, M.G., Eady, R.R. & Postgate, J.R. (1976) In: Proceedings of the First International Symposium on Nitrogen Fixation (Newton, W.E. & Nijman, C.J., eds.) vol. I, pp. 150-176, Washington State University Press, Pullman.
25. Thorneley, R.N.F. & Lowe, D.J. (1983) Biochem.J. 215, 393-403.
26. Burgess, B.K., Jacobs, D.B. & Stiefel, E.I. (1980) Biochim.Biophys.Acta 614, 196-202.
27. Hageman, R.V. & Burris, R.H. (1978) Biochemistry 17, 4117-4124.
28. Tso, M.-Y.W., Ljones, T. & Burris, R.H. (1972) Biochim.Biophys.Acta 267, 600-604.
29. Lowe, D.J. & Thorneley, R.N.F. (1984) Biochem.J. 224, 877-886.
30. Hageman, R.V., Orme-Johnson, W.H. & Burris, R.H. (1980) Biochemistry, 19, 2333-2342.
31. Ljones, T. & Burris, R.H. (1978) Biochemistry 17, 1866-1872.
32. Mortenson, L.E. & Thorneley, R.N.F. (1979) Ann.Rev.Biochem. 48, 387-418.
33. Burgess, B.K. (1984) In: Advances in Nitrogen Fixation Research (Veeger, C. & Newton, W.E. eds.) pp. 103-114, Nijhoff, Junk, Pudoc, The Hague.
34. Dunham, W.R., Hagen, W.R., Braaksma, A., Grande, H.J. & Haaker, H. (1985) Eur.J.Biochem. 146, 497-501.
35. Shah, V.K. & Brill, W.J. (1977) Proc.Natl.Acad.Sci. USA 74, 3249-3253.
36. Münck, E., Rhodes, H., Orme-Johson, W.H., Davis, L.C., Brill, W.J. & Shah, V.K. (1975) Biochim.Biophys.Acta 400, 32-53.
37. Robson, R.L., Eady, R.R., Richardson, T.H., Miller, R.W. & Hawkins, M. (1986) Nature 322, 388-390.
38. Braaksma, A., Haaker, H. & Veeger, C. (1983) Eur.J.Biochem. 133, 71-76.
39. Haaker, H., Cordewener, J., Ten Asbroek, A., Wassink, H., Eady, R. & Veeger, C. (1985) In: Nitrogen fixation research progress (Evans, H.J., Bottomley, P.J. & Newton, W.E., eds.) pp. 567-576, Martinus Nijhoff.
40. Eady, R.R. (1980) Methods Enzymol. 69, 753-792.
41. Lindahl, P.A., Orme-Johnson, W.H., Kent, T.A., Day, E.P. & Münck, E. (1985) Rev.Port.Quim. 27, 191-193.
42. Hagen, W.R., Eady, R.R., Dunham, W.R. & Haaker, H. (1985) FEBS Lett. 189, 250-254.
43. Burris, R.H. & Orme-Johnson, W.H. (1976) In: Proceedings of the First International Symposium on Nitrogen Fixation (Newton, W.E. & Nyman, C.J.,

eds.) vol. I pp. 208-233, Washington State University Press, Pullman.
44. McKenna, C.E., Huang, C.W., Jones, J.B., McKenna, M-C., Nakajima, T. & Nguyen, H.T. (1980) In: Nitrogen Fixation (Newton, W.E. & Orme-Johson, W.H. eds.) vol. I, pp. 223-236, University Park Press, Baltimore, M.D.
45. Hageman, R.V. & Burris, R.H. (1980) Biochim.Biophys.Acta. 591, 63-75.
46. Thorneley, R.N.F. & Lowe, D.J. (1981) In: Current Perspectives in Nitrogen Fixation (Gibson, A.H. & Newton, W.E. eds.) p. 360, Austr.Acad.Sci. Canberra.
47. Thorneley, R.N.F. & Lowe, D.J. (1982) Israel J.Bot. 31, 61-71.
48. Ljones, T. & Burris, R.H. (1972) Biochim.Biophys.Acta 8, 5103-5108.
49. Watt, G.D., Bulen, W.A., Burns, A. & Hadfield, K.L. (1975) Biochemistry 14, 4266-4272.
50. Bui, P.T. & Mortenson, L.E. (1969) Biochemistry 8, 2462-2465.
51. Hadfield, K.L. & Bulen, W.A. (1969) Biochemistry 8, 5103-5108.
52. Jeng, D.Y., Morris, J.A. & Mortenson, L.E. (1970) J. Biol.Chem. 245, 2809-2813.
53. Mortenson, L.E., Walker, M.N. & Walker, G.A. (1976) In: Proceedings of the First International Symposium on Nitrogen Fixation, (Newton, W.E. & Nijman, C.J., eds.) vol.I, pp. 117-149, Washington State University Press, Pullman.
54. Iman, S. & Eady, R.R. (1980) FEBS Lett. 110, 35-38.
55. Orme-Johnson, W.H., Hamilton, W.D., Ljones, T., Tso, M.-Y.W., Burrio, R.H., Shah, V.K.& Brill, W.J. (1972) Proc. Natl. Acad. Sci USA 69, 3142-3145.
56. Smith, B.E., Lowe, D.J. & Bray, R.C. (1973) Biochem.J. 135, 331-341.
57. Yates, M.G., Thorneley, R.N.F. & Lowe, D.J. (1975) FEBS Lett. 60, 89-93.
58. Thorneley, R.N.F., Yates, M.G. & Lowe, D.J. (1976) Biochem.J. 155, 137-144.
59. Watt, G.D. (1985) In: Nitrogen fixation research progress (Evans, H.J., Bottomley, P.J. & Newton, W.E., eds.) pp. 585-590, Martinus Nijhoff.
60. Braaksma, A., Haaker, H., Grande, H.J. & Veeger, C. (1982) Eur. J.Biochem. 121, 483-491.
61. Morgan, T.V., Prince, R.C. & Mortenson, L.E. (1986) FEBS Lett. 206, 4-8.
62. Zumft, W.G., Mortenson, L.E. & Palmer, G. (1974) Eur. J. Biochem. 46, 525-535.
63. Orme-Johnson, W.H. & Davis, L.C. (1977) In : Iron-Sulfur Proteins III (Lovenberg, W., ed.) pp. 16-60, Academic Press, New York.
64. Ljones, T. & Burris, R.H. (1978) Biochem.Biophys. Res. Commun. 80, 22-25.
65. Braaksma, A. (1985) Ph. D. Thesis, Landbouwuniversiteit, Pudoc, Wageningen.
66. Thorneley, R.N.F. (1975) Biochem. J. 145, 391-396.
67. Thorneley, R.N.F. & Cornish-Bowden, A. (1977) Biochem. J. 165, 255-262.

68. Lowe, D.J. & Thorneley, R.N.F. (1984) Biochem. J. 224, 895-901.
69. Ljones, T. & Burris, R.H. (1972) Biochim. Biophys. Acta 275, 93-101.
70. Bergersen, F.J. & Turner, G.L. (1973) Biochem. J. 131, 61-75.
71. Emerich, D.W. & Burris, R.H. (1976) Proc. Natl. Acad. Sci USA 73, 4369-4373.
72. Thorneley, R.N.F., Eady, R.R. & Yates, M.G. (1975) Biochim. Biophys. Acta 403, 269-284.
73. Zumft, W.G., Palmer, G.& Mortenson, L.E. (1973) Biochim. Biophys. Acta, 292, 413-421.
74. Tso, M.-Y.W. & Burris, R.H. (1973) Biochim. Biophys. Acta 309, 263-270.
75. You, C.B., Wang, H.X., Gao, M.S. & Ping, S.Z. (1984) In: Advances in Nitrogen Fixation Research (Veeger, C. & Newton, W.E., eds) p 168.
76. Orme-Johnson, W.H. (1985) Ann. Rev. Biophys. Biophys. Chem. 14, 419-459.
77. Hawkes, T.R., Mclean, P.A. & Smith, B.E. (1984) Biochem. J. 217, 317-321.
78. Smith, B.E. (1983) In: Nitrogen Fixation : The Chemical-Biochemical-Genetic Interface (Muller, A. & Newton W.E., eds) pp. 23-62, Denum Press, New York.
79. Li, J-G., Burgess, B.K. & Corbin, J.L. (1982) Biochemistry 21, 4393-4402.
80. Leigh, G.J. (1977) In : Recent Developments in Nitrogen Fixation (Newton, W.E., Postgate, J.R. & Rodriguez-Barrueco, C. (eds.) pp. 1-24, Academic Press, London.
81. Davis, L.C. (1980) Arch. Biochem. Biophys. 204, 270-276.
82. Winter, H.C. & Burris, R.H. (1976) Ann. Rev. Biochem. 45, 409-426.
83. Smith, B.E., Thorneley, R.N.F., Eady, R.R. & Mortenson, L.E. (1976) Biochem.J. 157, 439-447.
84. Emerich, D.W., Ljones, T. & Burris, R.H. (1978) Biochim. Biophys. Acta 527, 359-369.
85. Eady, R.R., Lowe, D.J. & Thorneley, R.N.F. (1978) FEBS Lett. 95, 211-213.
86. Eady, R.R., Kennedy, C., Smith, B.E., Thorneley, R.N.F., Yates, G. & Postgate, J.R. (1975) Biochem. Soc. Trans. 3, 488-492.
87. Stiefel, E.I. (1977) In: Recent Developments in Nitrogen Fixation (Newton, W.E., Postgate, J.R. & Rodriguez-Barrueco, eds.) pp. 69-108, Academic Press, London.
88. Haaker, H. & Veeger, C. (1984) Trends Biochem.Sci. 9, 188-192.
89. Robinson, J.D. & Flashner, M.S. (1979) Biochem. Biophys. Acta 549, 146-176.
90. Jorgenson, P.L. (1982) Biochim. Biophys. Acta 694, 27-68.
91. de Meis, L. & Vianna, A.L. (1979) Ann. Rev. Biochem. 48, 275-292.
92. Socho, G., Wallmark, B., Saccomani, G. Rabon, E., Stewart, H.B., Dibona,

D.R. & Berglindh, T. (1982) Curr. Top. Memb. Transp. 16, 135-160.
93. Goffean, A. & Slayman, C.W. (1981) Biochim. Biophys. Acta 639, 197-223.
94. Kagawa, Y. (1978) Adv. Biophys. 10, 209-247.
95. Wilson, D.B. & Smith, J.B. (1978) In: Bacterial Transport (Rosen, B.P., ed.) pp. 495-557, Marcel Dekker, Inc., New York.
96. Penefsky, H.S. (1979) Adv. Enzymol. 49, 223-280.
97. Ferguson, S.J. & Sorgato, M.C. (1982) Ann. Rev. Biochem. 52, 185-217.
98. Shavit, N. (1980) Ann. Rev. Biochem. 49, 111-138.
99. Mitchell, P. (1961) Nature 191, 144-148.
100. Foster, D.L. & Fillingame, R.H. (1979) J. Biol. Chem. 254, 8230-8236.
101. Pick, U. & Racker, E. (1979) J. Biol. Chem. 254, 2793-2799.
102. Glaser, E., Norling, B. & Ernster, L. (1980) Eur. J. Biochem. 110, 225-235.
103. Criddle, R.S., Johnston, R.F. & Stack, R.J. (1979) Curr. Top. Bioenerg. 9, 89-145.
104. Kagawa, Y, Sone, N., Hirata, H. & Yoshida, M. (1979) J. Bioenerg. Biomemb. 11, 39-78.
105. Stutterheim, E., Henneke, M.A.C. & Berden, J.A. (1980) Biochim. Biophys. Acta 592, 415-430.
106. Senior, A.E. & Wise, J.G. (1983) J. Membr. Biol. 73, 105-124.
107. Cross, R.L. & Nalin, C.M. (1982) J. Biol. Chem. 257, 2874-2881.
108. Penefsky, H.S. (1977) J. Biol. Chem. 252, 2891-2899.
109. Hatefi, Y. (1985) Ann. Rev. Biochem. 54, 1015-1069.
110. Mitchell, P. (1977) Ann. Rev. Biochem. 46, 996-1005.
111. Kwiki, Y., Halsey, J., Biltonen, R. & Racker, E. (1976) Biochemistry 15, 4956-4961.
112. Webb, M.r., Grubmeyer, C., Penefsky, H.S. & Trentham, D.R. (1980) J. Biol. Chem. 255, 11637-11639.
113. Mortenson, L.E., Webb, M., Bare, R., Cramer, S.P. & Morgan, T.V. (1985) In: Nitrogen fixation research progress (Evans, H.J., Bottomley, P.J. & Newton, W.E., eds.) pp. 577-583, Martinus Nijhoff.
114. Slater, E.C. (1974) In: Dynamics of Energy-Transducing Membranes (Ernster, L., Estabrook, R.W. & Slater, E.C., eds) pp. 1-20, Elsevier, Amsterdam.
115. Harris, D.A., Rosing, J., van de Stadt, R.J. & Slater, E.C. (1973) Biochim. Biophys. Acta 314, 149-153.
116. Hutton, R.L. & Boyer, P.D. (1979) J. Biol. Chem. 254, 9990-9993.
117. Boyer, P.D., Cross, R.L. & Momsen, W. (1973) Proc. Natl. Acad. Sci USA 70, 2837-2839.
118. Gresser, M.J., Myers, J.A. & Boyer, P.D. (1982) J. Biol. Chem. 257,

12030-12038.
119. Kayalar, C., Rosing, J. & Boyer, P.D. (1977) J. Biol. Chem. 252, 2486-2491.
120. Harris, D.A. (1978) Biochim.Biophys.Acta 463, 245-273.

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Chapter 2
Binding of MgATP to the Nitrogenase Proteins from Azotobacter vinelandii

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Binding of MgATP to the MoFe and Fe proteins from *Azotobacter vinelandii* has been studied. By means of the flow dialysis technique it was demonstrated that one molecule of reduced Fe protein binds one molecule of MgATP, with a dissociation constant of 0.56 ± 0.11 mM. The oxidized Fe protein binds two molecules of MgATP, with identical intrinsic dissociation constants of 0.29 ± 0.05 mM. The binding of MgATP to the Fe protein was also studied by equilibrium dialysis. It was found that during dialysis of reduced Fe protein in the presence of MgATP, dithionite was oxidized. Moreover, in the presence of MgATP both reduced and oxidized Fe protein were inactivated during the dialysis. These observations demonstrate that binding of MgATP to the Fe protein can only be measured by a relatively fast method.

With the same methods as used for the Fe protein, no binding of MgATP to the MoFe protein of *A. vinelandii* could be demonstrated.

The redox properties of the Fe protein in the presence and absence of MgATP are discussed with respect to the observed binding properties of MgATP for the Fe protein. The implications of these results are discussed with respect to the present models for the interactions between the Fe and MoFe proteins of nitrogenase.

Nitrogenase is the enzyme system that, in an anaerobic environment, catalyses the reduction of N_2 to ammonia when supplied with MgATP and a low-potential electron donor. Nitrogenase consists of two easily separable proteins: a tetrameric MoFe protein probably carrying the substrate-reducing site and a dimeric Fe protein [1 – 3]. Data obtained from cluster extrusion [4, 5] and chiroptical spectroscopy [6] indicate the presence of an [4 Fe-4 S] cluster in the Fe protein. Recently, Braaksma et al. [7] reported from redox-dependent EPR measurements that the Fe protein isolated from *Azotobacter vinelandii* is able to accommodate two reducing equivalents.

Reduction of N_2 to NH_3, acetylene to ethylene and protons to H_2, is accompanied by the hydrolysis of ATP to ADP and orthophosphate. The specific role of ATP in nitrogenase-catalyzed reductions is not known, although several hypotheses have been offered [8 – 10]. It has been demonstrated that ATP hydrolysis is coupled to the electron transfer between the two nitrogenase proteins [10, 11]. In the absence of reducing agents, ATP is hydrolysed at a diminished rate by the nitrogenase complex but not by the individual components [12 – 14].

The interactions of ATP with the nitrogenase proteins from different organisms has been studied. Binding of MgATP to the Fe protein has been reported [15 – 19]. Binding of MgATP to the MoFe protein has only been found for the MoFe protein isolated from *Klebsiella pneumoniae* [16, 20]. The binding data obtained by column gel filtration indicated four binding sites for MgATP on Kp_1 [20]. Using a gel equilibration method, Tso and Burris [17] found that Cp_2 binds two molecules of MgATP. But these experiments were reported not to be reproducible [1]. Also indirect methods have been used to study the binding of MgATP to the Fe protein, such as EPR spectroscopy [19], as well as the reaction rate of the iron chelators 2,2'-bipyridyl and bathophenanthrolinedisulfonate (BPS) with the Fe protein – MgATP complex [1, 13, 21]. The EPR data obtained by Zumft et al. [19] indicated two binding sites for MgATP on Cp_2, but their results have been criticized by Eady and Smith [22]. The experiments with the iron chelators showed an S-shaped dependence of the reaction velocity on the ATP concentration, which led to the conclusion that two molecules of ATP are involved.

Binding studies of ATP to the complex between the MoFe and Fe protein are complicated since nitrogenase hydrolyses ATP in the absence of an electron donor. Information about the interaction of ATP with the nitrogenase complex is derived from steady-state and pre-steady-state kinetics. A kinetic dependence on two molecules of MgATP for the H_2 evolution reaction has been demonstrated [10, 23, 24]. From pre-steady-state kinetics Hageman et al. [10] concluded that two molecules of MgATP are required for the initial electron transfer from Av_2 to Av_1, while experiments of Thorneley [25] indicated that only one molecule of MgATP is involved in electron transfer from Kp_2 to Kp_1.

Most procedures for quantitative studies of ligand binding to proteins are not suitable for the nitrogenase proteins, due to their lability and O_2 sensitivity. In this study flow dialysis and equilibrium dialysis have been applied to study binding of MgATP to the separate nitrogenase proteins. Complications that arise during binding experiments, like inactivation and oxidation of the Fe protein under fully anaerobic conditions, have been examined. Binding properties of MgATP were determined for both the reduced and oxidized Av_2 protein and for the Av_1 protein as isolated. The implications of these binding properties with respect to the model for the nitrogenase reaction will be discussed.

Abbreviations. The MoFe and Fe proteins of the nitrogenases of *Azotobacter vinelandii*, *Clostridium pasteurianum* and *Klebsiella pneumoniae* are referred to as Av_1 and Av_2, Cp_1 and Cp_2 and Kp_1 and Kp_2, respectively; EPR, electron paramagnetic resonance; BPS, bathophenanthrolinedisulfonate; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-amino}ethane sulfonic acid; SEE, standard error of estimates.

MATERIALS AND METHODS

Growth Conditions and Enzyme Preparation

Azotobacter vinelandii ATCC 478 was grown in a batch culture of 250 l, harvested during the logarithmic phase and stored at −70 °C [7]. The component proteins were purified as described by Braaksma et al. [7] and had specific activities of about 2500 nmol C_2H_4 formed · min^{-1} · (mg MoFe protein)$^{-1}$ and about 2200 nmol C_2H_4 formed · min^{-1} · (mg Fe protein)$^{-1}$ when measured under standard assay conditions at pH 7.4 and optimum levels of the complementary protein. The Fe protein was oxidized by the addition of 1 M Na_2SO_3 (pH 7.4) and was desalted by passing the protein over a Sephadex G-25 column equilibrated with 25 mM Tes/NaOH, 2 mM Na_2SO_3, 5 mM $MgCl_2$, final pH 7.4.

Analytical Methods

The nitrogenase activity (acetylene reduction) was measured in the assay system as described earlier [7]. Protein concentrations were measured by the Coomassie brilliant blue method [26] with bovine serum albumin as standard. For the calculation of the molar nitrogenase protein concentration we used for the MoFe protein M_r = 220000 and for the Fe protein M_r = 60000 [7, 27, 28].

The dithionite concentration of a nitrogenase-containing solution was measured by its ability to reduce benzyl viologen. Cuvettes for anaerobic spectrophotometry (1-cm light path) containing 1 ml 50 mM Tes/NaOH, 20 mM α-D-glucose, 0.1 mM benzyl viologen, final pH 7.4 were stoppered with a Subaseal rubber cap and made anaerobically by five cycles of evacuation and filling with argon gas. Glucose oxidase to a final concentration of 0.2 mg/ml was then added by a syringe, followed 5 min later by the dithionite solution. The reduction of benzyl viologen was determined at 540 nm, using an absorption coefficient of 13.05 $mM^{-1} \cdot cm^{-1}$ [29].

Equilibrium Dialysis

Equilibrium dialysis experiments were carried out in a Dianorm dialyzer consisting of Teflon cells with a volume of 0.5 ml. A semipermeable membrane separates the dialysis cell into two halves. The membranes were cut out of Spectra/Por 2 dialysis tubing which had been boiled previously in water containing EDTA. After flushing the cells with argon, 0.2 ml of a nitrogenase protein solution was injected into one half-cell and 0.2 ml of ligand solution was added to the other half-cell. The dialyzer was rotated at 20 rev./min at room temperature. Control experiments with bovine serum albumin showed that equilibrium was attained within 4 h. At that time three 25-µl samples were removed from both ligand and protein compartments for the measurement of the ATP, ADP and AMP concentrations [30]. From the difference in ligand concentrations in the two half-cells the values of mol ligand bound/mol of protein were calculated.

Flow Dialysis

The flow dialysis technique we used was originally developed by Colowick and Womack [31]. The dialysis rate cell consists of an upper and a lower chamber, separated by a semipermeable membrane. The capacities of the upper and lower chamber are 0.8 ml and 0.6 ml respectively, and the diffusion area is 0.5 cm^2. The upper chamber has an opening which can be closed with a Subaseal cap to make it anaerobic by flushing with argon. Through the lower chamber argon-flushed 25 mM Tris/HCl (pH 7.4) was pumped at a constant rate of 0.5 ml/min. After filling the upper chamber with 0.2 ml of a nitrogenase-component-containing solution, a fixed concentration of [α-^{32}P]ATP was introduced into the protein solution. The effluent was sampled for measurement of radioactivity. After about 5 min a steady-state level of radioactivity in the lower chamber was reached. 10 min after the addition of labeled ATP to the protein, excess unlabeled ATP was added to the upper chamber. The radioactivity in the effluent then reaches a maximum value. The fraction of labeled ATP present in the free state, before dilution with unlabeled ATP, was calculated by dividing the amounts of radioactivity in the effluent before and after dilution. By this procedure it is possible to measure reproducibly the fraction of ligand bound to protein. Under the conditions employed, the loss of ATP by dialysis from the upper chamber was less than 10 % in 15 min. Corrections were made for the small changes in ATP concentration due to loss by dialysis.

Reaction between Fe Protein and Bathophenantrolinedisulfonate

For measurement of the rate of reaction of bathophenantrolinedisulfonate (BPS) with Av_2 at different concentrations of MgATP, the method described by Ljones and Burris [21] was used. For the absorption coefficient of the Fe^{2+}-BPS complex, we used ε_{535} = 22.14 $mM^{-1} \cdot cm^{-1}$ [21].

Analysis of Data

The data obtained from flow dialysis were fitted to the equation:

$$r = \frac{N[\mathrm{MgATP}]}{K + [\mathrm{MgATP}]} \tag{1}$$

where r is mol MgATP bound/mol protein, N the number of binding sites and K the dissociation constant. The method of linear least squares was employed to obtain the values for N and K. The data obtained from the BPS measurements were fitted to the equation as derived according to the King and Altman procedure [32] for the reaction as shown in Scheme 1. This leads to:

$$v = \frac{c_1[\mathrm{MgATP}] + c_2[\mathrm{MgATP}]^2}{c_3 + c_4[\mathrm{MgATP}] + [\mathrm{MgATP}]^2} \tag{2}$$

where v is the velocity of complex formation of BPS with Fe^{2+} and $c_1 - c_4$ are constants that include the different rate constants given in Scheme 1. The BPS data also were fitted to the equation derived by Hageman et al. [10]:

$$v = \frac{V[\mathrm{MgATP}]^2}{K_A K_B + K_B[\mathrm{MgATP}] + [\mathrm{MgATP}]^2}. \tag{3}$$

Computer fits were obtained by adjusting the parameters of the theoretical functions with a weighted least-squares iterative procedure to the experimental data. The standard error of estimates (SEE) as a percentage is derived as

$$100 \times \sqrt{\sum_i \left(\frac{y_b - y_i}{y_b}\right)^2 / n} \tag{4}$$

in which y_b is, for example, the calculated velocity at a certain MgATP concentration, using the parameter values as obtained by computer fitting, and y_i the experimentally determined velocity. n is the number of experimental points.

Chemicals and Gasses

[α-^{32}P]ATP (760 Ci/mmol) was obtained from New England Nuclear (NEN). All other (bio)chemicals were analytical grade obtained from commercial sources. Enzymatic analysis indicated that the ATP used contained about 4% ADP and about 2% AMP. Argon was purified by passage over hot (150°C) BASF catalyst.

RESULTS

Flow dialysis, a non-equilibrium dialysis technique, was used to study the interaction of MgATP with the nitrogenase proteins. This technique is based upon the fact that the rate of diffusion of the ligand across the membrane from the protein-containing side is proportional to the concentration of unbound ligand in this chamber. Titration of a single protein sample with increasing amounts of ligand yields a complete binding curve [31]. In the present study with the labile nitrogenase proteins the most reproducible results were obtained with the procedure described in Materials and Methods. In Fig. 1, a typical experiment is shown measuring binding of [α-^{32}P]ATP by 230 μM oxidized Av_2. Within 6 min after the addition of radioactive ATP (0.1 mM), the diffusion rate across the dialysis membrane is constant and measured accurately. The total concentration of radioactive ATP in the protein compartment was measured 6 min after the addition of excess unlabeled ATP (9.6 mM). In this way it was possible to measure accurately the amount of ATP bound to Av_2 at different MgATP concentrations. Binding of MgATP to oxidized and reduced Av_2 was studied and the results are presented in Fig. 2. Oxidation of Av_2 was performed in two different ways: one, Na_2SO_3 oxidation [7, 29] and the other physiological oxidation by a catalytic amount of Av_1 in the presence of MgATP and an ATP-regenerating system. Both methods of oxidation resulted in the same binding data. During flow dialysis experiments aliquots were taken from the protein compartment to check the specific activity of Av_2. Only in the case of Av_2^{ox} was a slight inactivation (10–20%) observed routinely during the essential time of dialysis (the first 10 min). When flow dialysis was continued for another 10 min the specific activity of Av_2^{ox} dropped to 50% but no significant increase in binding was observed. The binding data obtained for oxidized and reduced Av_2 were plotted according to Scatchard [33] and were fitted by straight lines (Fig. 2B). The binding parameters obtained in this way for reduced Av_2 are: $N = 1.3 \pm 0.3$ and $K^r = 0.56 \pm 0.11$ mM, for oxidized Av_2: $N = 2.2 \pm 0.4$ and $K_i^o = 0.29 \pm 0.05$ mM, with K^r = dissociation constant of MgATP from reduced Av_2 and K_i^o = intrinsic dissociation constant of MgATP from oxidized Av_2.

Flow dialysis was carried out with 100 μM Av_1 in 25 mM Tes/NaOH, 2 mM $Na_2S_2O_4$, 5 mM $MgCl_2$ and 0.1 mM or 1 mM MgATP, final pH 7.4. Within the experimental error of the technique (≈ 5%) no binding of MgATP to Av_1 could be detected (not shown). This means that in the presence of 1 mM MgATP less than 0.5 mol MgATP are bound/mol Av_1. This in contrast to Kp_1 where at 1 mM MgATP 2.4 mol MgATP are bound/mol Kp_1 [20].

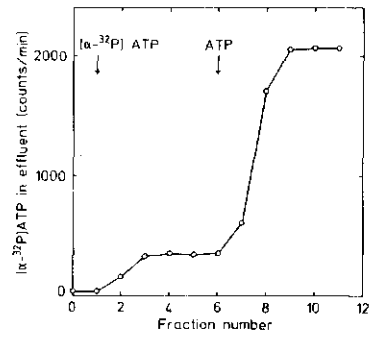


Fig. 1. *Binding of MgATP to Av_2 as determined by flow dialysis.* Flow dialysis was performed as described in Materials and Methods. At the first arrow, 10 μl of 2 mM [α-^{32}P]MgATP (≈ 1.5×10^5 counts/min) were added to the upper chamber, which was supplied with 0.19 ml of 230 μM Av_2 in 25 mM Tes/NaOH, 2 mM Na_2SO_3, 5 mM $MgCl_2$, final pH 7.4. 10 min later 10 μl of 0.2 M unlabeled MgATP was added to the upper chamber (second arrow). 1-ml fractions of eluate were collected directly into counting vials and radioactivity was measured by using a Packard Tri-Carb 2450 liquid scintillation counter

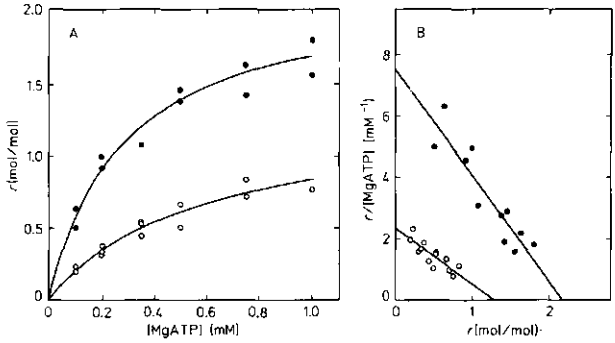


Fig. 2. *Binding of MgATP to reduced and oxidized Av_2 as determined by flow dialysis.* Experimental conditions as described in the legend to Fig. 1 for the oxidized protein, while for the reduced protein 2 mM $Na_2S_2O_4$ was used instead of 2 mM Na_2SO_3. Protein concentrations varied over 100–170 μM. r is mol ATP bound/mol Av_2. (A) Binding data obtained for reduced (○——○) and oxidized (●——●) Av_2. (B) Binding data plotted according to Scatchard. The straight lines were fitted with $N = 1.3 \pm 0.3$ and $K^r = 0.56 \pm 0.11$ mM for reduced Av_2 (○——○) and $N = 2.2 \pm 0.4$ and $K_i^o = 0.29 \pm 0.05$ mM for oxidized Av_2 (●——●)

Table 1. *Equilibrium dialysis of Av_1 and Av_2 against different concentrations of MgATP*

Data were obtained from equilibrium dialysis experiments as described in Materials and Methods. 100 μM Av_1 in 25 mM Tes/NaOH, 2 mM $Na_2S_2O_4$, final pH 7.4 was dialysed against 25 mM Tes/NaOH, 2 mM $Na_2S_2O_4$, 0.6 M NaCl, 0.1 mM bovine serum albumin (BSA), 9 mM $MgCl_2$ and the indicated ATP concentrations, final pH 7.4. 46 μM Av_2 in 25 mM Tris HCl, 2 mM $Na_2S_2O_4$, 5 mM $MgCl_2$, final pH 7.4 was dialysed against 25 mM Tes NaOH, 2 mM $Na_2S_2O_4$, 0.6 M NaCl, 0.1 mM BSA and the indicated ATP concentrations, final pH 7.4

Protein present in protein compartment	Initial ATP concen in ligand compartment	Nucleotide concentrations after 4 h of dialysis in protein compartment			ligand compartment		
		ATP	ADP	AMP	ATP	ADP	AMP
	mM						
100 μM Av_1	1.5	0.66	0.04	0.02	0.64	0.07	0.01
100 μM Av_1	0.75	0.32	0.02	0.02	0.33	0.04	0.02
46 μM Av_2	2.2	1.07	0.04	0.04	0.97	0.06	0.05
46 μM Av_2	1.0	0.48	0.03	0.01	0.40	0.04	0.02
100 μM BSA	1.9	0.90	0.04	0.02	0.90	0.03	0.01

Another technique to study binding of a ligand to a protein is equilibrium dialysis. Since we attempted to demonstrate binding of the negatively charged ligand $MgATP^{2-}$ to the negatively charged nitrogenase proteins, it was important to minimize the Donnan potential. Therefore nitrogenase protein was dialysed against a solution of bovine serum albumin and in the presence of 0.3 M NaCl. Equilibrium dialysis data obtained in this way are shown in Table 1. With this method again we could not detect any binding of MgATP to the Av_1 protein (Table 1). Also when NaCl was omitted from the solutions to avoid ionic inhibition of MgATP binding to Av_1, no binding was observed. When on the other hand Av_2 was dialysed against MgATP binding of MgATP was observed (Table 1).

From the data of Table 1 it is clear that the concentrations of ADP and AMP present after 4 h of dialysis of Av_1 or Av_2 against MgATP do not differ considerably from those in the control experiment with albumin present on both sides of the dialysis membrane. From these results and from direct activity measurements, it was clear that the Av_1 and Av_2 protein preparations used did not contain any significant ATPase or myokinase activity. Furthermore, the ADP and AMP present at the end of the dialysis experiments was within the experimental error equally distributed between the dialysis compartments of protein and ligand solution, indicating no specific interactions with the nitrogenase proteins under these conditions. Fig. 3 represents the binding curve for MgATP to reduced Av_2 obtained from six separate equilibrium dialysis experiments. Because of the scattering in the binding data, we examined the stability of the Fe protein during dialysis. Initially oxidized Av_2 is very labile (Fig. 4A) in the presence of MgATP. But reduced Av_2 is also not stable in the presence of MgATP. In a control experiment without MgATP, reduced Av_2 was stable for at least 4 h. When under identical conditions the dithionite concentration was measured, it was found that in the absence of MgATP, the $Na_2S_2O_4$ concentration slowly decreased, but its concentration was reduced to zero in the presence of MgATP (Fig. 4B). Similar observations were reported by Stephens et al. [6]. To exclude the possibility that dithionite was used in the

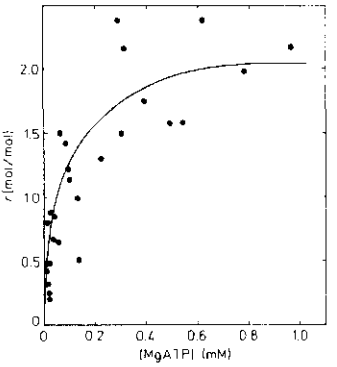


Fig. 3. *Binding curve of MgATP to reduced Av_2 as determined by equilibrium dialysis.* Experimental conditions are described in the legend of Table 1. Protein concentration was 46 μM. Data were obtained from six separate dialysis experiments

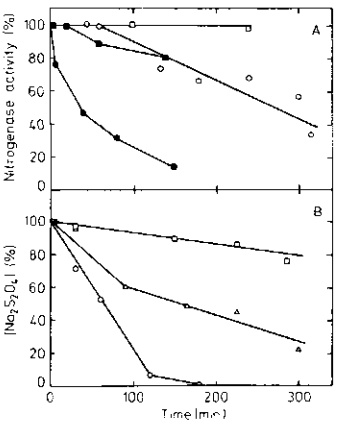


Fig. 4. *The effect of MgATP on the specific activity of Av_2 and on the dithionite concentration.* The specific activity of Av_2 and the dithionite concentration were measured as described in Materials and Methods. Av_2 was oxidized with Na_2SO_3 as described in Materials and Methods. 100% activity was 2200 nmol C_2H_4 formed · min^{-1} · mg Av_2^{-1}. The protein was incubated as indicated and at various time intervals, samples were withdrawn for measurements of the specific activity of Av_2 (A) and the dithionite concentration (B). (A) (○ ○) 70 μM Av_2, 25 mM Tes/NaOH, 5 mM $MgCl_2$, 1 mM ATP and 2 mM $Na_2S_2O_4$, final pH 7.4; (□ □) 70 μM Av_2, 25 mM Tes/NaOH, 5 mM $MgCl_2$, 2 mM $Na_2S_2O_4$, final pH 7.4; (■ ■) 70 μM Av_2, 25 mM Tes/NaOH, 5 mM $MgCl_2$, 2 mM Na_2SO_3, final pH 7.4; (● ●) same plus 1 mM ATP. (B) (○ ○) 70 μM Av_2, 25 mM Tes/NaOH, 5 mM $MgCl_2$, 1 mM ATP, initially 2 mM $Na_2S_2O_4$, final pH 7.4; (△ △) same with initially 5 mM $Na_2S_2O_4$; (□ □) 70 μM Av_2, 25 mM Tes/NaOH, 5 mM $MgCl_2$, 2 mM $Na_2S_2O_4$, final pH 7.4

nitrogenase reaction, we ascertained that there was no Av_1 impurity present in the Av_2 preparation used. No ATPase, H_2 production or acetylene reduction activity could be detected in the Av_2 solution. The results of Fig. 4 illustrate that during equilibrium dialysis in the presence of MgATP dithionite is consumed and the Fe protein is partly inactivated. It was found that these partly inactivated Av_2 preparations still bind MgATP, which at ATP concentrations around 1 mM resulted

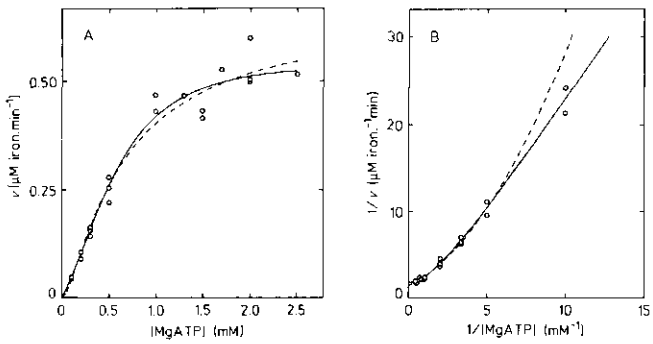


Fig. 5. *MgATP-dependent reaction of Av_2 with bathophenantrolinedisulphonate.* Reaction conditions were 50 mM Tes/NaOH, 4 mM $MgCl_2$, 0.1 mM bathophenantrolinedisulphonate, 5 mM $Na_2S_2O_4$, 10 mM creatine phosphate, 0.1 mg/ml creatine kinase and 6 μM Av_2, final pH 7.4. The reaction was started by the addition of MgATP and the initial rate of complex formation was measured following the absorbance increase at 535 nm. v is expressed in μM iron complexed per min, using an absorption coefficient of 22.14 $mM^{-1} \cdot cm^{-1}$ for the Fe^{2+}-BPS complex. (A) v vs [MgATP]; (——) data fitted according to Eqn (2) (see Materials and Methods), with $c_1 = 0.22$, $c_2 = 0.53$, $c_3 = 0.60$ and $c_4 = 0.21$, SEE = 8%; (- - -) data fitted according to Eqn (3), with $V = 0.69$, $K_A = 0.2$ and $K_B = 0.6$, SEE = 11%. (B) Double-reciprocal plot of A; (——) Eqn (2); (- - - -) Eqn (3)

in an ATP/Av_2 ratio even greater than two if the Av_2 concentration is based on the remaining activity. It is very tempting to conclude that initially reduced Av_2 is oxidized during the dialysis and binds two molecules of MgATP, a similar result as obtained by flow dialysis. However, since the oxidation/reduction state and the specific activity of Av_2 is variable during a dialysis experiment, the results can only be used as indicative.

As a third method to study the binding of MgATP to the Fe protein we measured the initial velocity of the reaction of bathophenantrolinedisulfonate with the [4 Fe-4 S] site of Av_2 at different concentrations of MgATP. The results obtained in this way are presented in Fig. 5 and fitted to the equations given in Materials and Methods. It is clear that, especially at the lower MgATP concentrations, Eqn (2) gives a better fit than Eqn (3).

DISCUSSION

The binding of MgATP to Av_2 has been studied by flow dialysis. The results show that there is a distinct difference in binding properties of MgATP to reduced and oxidized Av_2. Reduced Av_2 has only one binding site for MgATP while oxidized Av_2 has two binding sites. The possibility arises that due to the enhanced lability of oxidized Av_2 in the presence of MgATP, the second binding site might be associated with inactive Av_2. From Fig. 4A it is clear that after the addition of MgATP oxidized Av_2 immediately starts to inactivate. During flow dialysis a 10–20% decrease in activity after 10 min of dialysis was routinely observed. When active oxidized Av_2 would have only one MgATP-binding site, to account for a total of two binding sites, the 20% inactive oxidized Av_2 must have at least five MgATP-binding sites. Also at longer dialysis incubation times, when the specific activity drops even more (up to 50%), no significant increase in binding was observed. These observations indicate to us that the increased binding of MgATP to oxidized Av_2 (see Fig. 2) is a property of the oxidized protein and not a property of the inactivated oxidized protein. It is known that dithionite-free Kp_2 may form a trimer or a tetramer in the presence of MgATP instead of the usual dimer [34]. The possibility arises that binding of MgATP to oxidized Av_2 induces the same phenomenon. The second MgATP binding site is generated after the formation of a more highly ordered Av_2 complex induced by binding of the first MgATP. We cannot exclude this possibility but in our experiments, when binding of MgATP was determined, the specific activity of oxidized Av_2 was still high. With our results on the stability of oxidized Av_2 plus MgATP (Fig. 4A), it is possible that during the centrifuge runs described by Thorneley and Eady [34] the dithionite-free Kp_2 was almost completely inactivated. It is therefore doubtful that the properties of the protein under the different conditions can be compared.

The binding properties of MgATP to reduced Av_2 might be more complex. We found it impossible to obtain binding data at MgATP concentrations higher than 1 mM. Therefore our results cannot exclude the possibility that reduced Av_2 binds a second molecule of MgATP. In this case, assuming negative cooperativity between the two binding sites of reduced Av_2, we estimate the dissociation constant of the second site to be larger than 2 mM. But since there is no evidence that reduced Av_2 binds more than one molecule of MgATP, we assume only one binding site in our calculations.

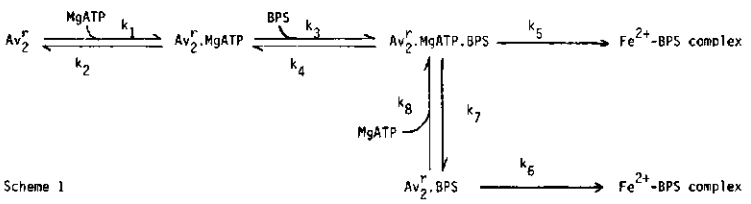
From the results obtained by equilibrium dialysis studies, one could conclude that two molecules of MgATP can bind to reduced Av_2. But we observed that Av_2 incubated with MgATP oxidizes its reductant in some way (Fig. 4B). Thus after 4 h of dialysis, the originally reduced Av_2 is probably partly in the oxidized state (see also Stephens et al. [6]). It is known that binding of MgATP to the Fe protein exposes the [4 Fe-4 S] center, as shown by complex formation of BPS with this cluster [10]. In this conformation the [4 Fe-4 S] cluster may be very labile and decomposes in time. Cermak and Smutek [35] studied the decomposition of dithionite in acid solutions and found that the decomposition is autocatalyzed by reactions of sulphide with dithionite. The decomposition products of the [4 Fe-4 S] cluster may therefore participate somehow in the oxidation reaction of dithionite. At this moment the detailed mechanism of the oxidation of dithionite is unclear.

Inactivation of Av_2 is not immediately reflected in a loss of ATP-binding capacity and might even increase the amount of MgATP bound to Av_2 (Fig. 3 and 4). When the binding of MgATP is corrected for the decrease in activity, values above

2 mol/mol can easily be obtained (not shown). But due to uncertainties in the oxidation state and activity of Av_2 it is clear that the binding studies of MgATP to Av_2 with equilibrium dialysis cannot be performed under strictly defined conditions and therefore the results are without meaning.

The reaction between BPS and the [4 Fe-4 S] cluster of the Fe protein in the presence of MgATP has been used to measure the binding constants of MgATP for the Fe protein [10, 21]. To explain the S-shaped dependence of the rate of reaction of BPS with Av_2, Hageman et al. [10] assumed the presence of two ATP-binding sites on reduced Av_2. Their results were fitted with an equation derived from a kinetic model in which both sites must be occupied by MgATP before there is a reaction of the Fe protein with BPS. However, the results of Hageman et al. [10] cannot exclude other models explaining the sigmoidal plots of the rate of the BPS reaction vs ATP concentration, such as the following.

(a) The reaction of BPS with the first Fe^{2+} of the cluster can change the Fe protein in such a way that a second ATP can bind to it, for instance as with oxidation of the protein. (b) The rate of reaction of BPS with the [4 Fe-4 S] site is relatively small compared with the rates of association and dissociation of MgATP with the Fe protein. Binding of MgATP to the Fe protein exposes the [4 Fe-4 S] cluster, so that BPS can interact with this site. When MgATP subsequently dissociates from the BPS-containing protein, the rate of reaction of BPS with the cluster (k_6) may be different from that (k_5) when MgATP is present (Scheme 1).

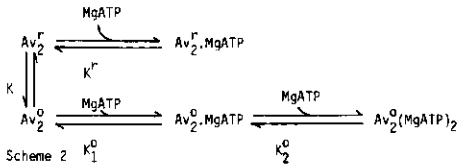


Scheme 1

For this model Eqn (2) was derived (see Materials and Methods). Fig. 5 shows that our results fit slightly better to Eqn (2), derived for only one MgATP-binding site, than to Eqn (3) which was derived for two MgATP-binding sites. Thus it is not justified to explain the sigmoidal curves of v vs [MgATP] exclusively by assuming two binding sites for MgATP on reduced Av_2.

Recently Miller et al. [20] showed with gel filtration that Kp_1 has four binding sites for MgATP. With the same method we were not able to detect any significant binding of MgATP to Av_1. We also tried equilibrium and flow dialysis without success to demonstrate MgATP binding to Av_1. The reason for this discrepancy might be higher dissociation constants, species differences of the proteins, or other reasons.

With EPR spectroscopy Braaksma et al. [7] showed that the midpoint potential of Av_2 in the absence of adenine nucleotides is -393 mV. When MgATP is bound to the protein, the midpoint potential undergoes a negative shift of 42 mV. Both in the presence and absence of MgATP the redox behaviour of Av_2 is that characteristic for a two-electron transferring protein. In the present study we determined the intrinsic binding constants of MgATP for reduced and oxidized Av_2 and the number of MgATP-binding sites for both oxidation states of the protein. With these data the shift in redox potential caused by the binding of MgATP to the Fe protein can be calculated (see Scheme 2). Scheme 2 is a thermodynamic repre-



Scheme 2

sentation of the equilibria between the different Av_2 species in the presence of MgATP. The equilibrium constants in this scheme are stoichiometric constants. For reduced Av_2 the intrinsic binding constant is equal to the stoichiometric constant $K^r = 0.56$ mM. In the case of oxidized Av_2 with two identical and independent binding sites the stoichiometric constants (K_1^o, K_2^o) can be obtained from the intrinsic binding constant (K_i^o) by a statistical factor as described by Klotz and Hunston [36]: $K_1^o = 0.5\,K_i^o = 0.145$ mM, and $K_2^o = 2K_i^o = 0.58$ mM. In Scheme 2 the redox reaction between $Av_2^r \cdot$ MgATP and $Av_2^o \cdot$ MgATP is omitted because in the thermodynamic description the ratio $Av_2^r \cdot MgATP/Av_2^o \cdot MgATP$ (K') is already determined by the three equilibria with equilibrium constants K^r, K and K_1^o, namely as follows:

$$K' = \frac{K \cdot K_1^o}{K^r}.$$

Therefore the concentrations of all species in Scheme 2 are determined by the four equilibrium constants as given in the scheme. For any applied potential we can calculate K from the Nernst equation for the free Av_2:

$$E_h = E_o' - \frac{RT}{nF} \ln K$$

in which $K = [Av_2^r]/[Av_2^o]$, $E_o' = -393$ mV and $n = 2$ [7]. Using Eqn (11) (see Appendix) and our binding data $K^r = 0.56$ mM, $K_1^o = 0.145$ mM and $K_2^o = 0.58$ mM, it can be calculated that at 5 mM MgATP

$$\frac{[Av_2^r \cdot MgATP]}{[Av_2]_0} = \frac{8.9\,K}{9.9\,K + 332.8}.$$

Substitution of K into this equation gives the fraction $Av_2^r \cdot$ MgATP at that particular potential. At infinitely low potential all Av_2 is in the reduced state and 90% is in the ATP form. At higher potentials the amount of $Av_2^r \cdot$ MgATP

declines. In this way the redox dependency of the intensity of the EPR signal can be calculated. This calculation gives a redox titration curve with $n = 2$ and a midpoint potential of -439 mV, which is close to the midpoint potential of -435 mV [7] found experimentally. Zumft et al. [37] reported a negative midpoint potential shift of 106 mV when MgATP binds to Cp_2. They noticed that the observed midpoint potential shift could be obtained by a 70-fold tighter binding of MgATP to oxidized than to reduced Cp_2. It was assumed that both oxidation states of Cp_2 bind two molecules of MgATP. On the other hand, assuming a similar difference in the number of binding sites of MgATP on oxidized and reduced Cp_2 as observed with Av_2, such a shift can be explained by the dissociation constant of MgATP for the oxidized protein being about 10-fold lower than for the reduced enzyme (5 μM vs 50 μM).

Braaksma et al. [7] showed that the nitrogenase activity correlates with the redox state of the free Fe protein, thus with the ratio $[Av_2^r]/[Av_2^o]$. During turnover of nitrogenase, free Av_2 will be in one of the forms shown in Scheme 2. At -390 mV, when the nitrogenase complex exhibits half of its maximum H_2 production capacity [7], one can calculate the relative amounts of each of the species given in Scheme 2: 0.2% Av_2^r, 2.1% $Av_2^r \cdot MgATP$, 0.3% Av_2^o, 10.1% $Av_2^o \cdot MgATP$ and 87.3% $Av_2^o \cdot (MgATP)_2$. In view of the excess of $Av_2^o(MgATP)_2$ present in comparison with the other forms, strong inhibition of nitrogenase-catalyzed reactions by MgATP can be expected, which is in fact not observed. This means that either Av_2^r or $Av_2^r \cdot MgATP$ or both must have at least a 10-fold higher affinity for Av_1 than Av_2^o has for MgATP in order to overcome this abortive type of inhibition by MgATP, assuming rapid equilibrium between Av_1 and Av_2^r and $Av_2^r \cdot MgATP$ and a rate-limiting step in product formation [7].

It is suggested that two molecules of MgATP are hydrolyzed per electron transferred from Av_2 to Av_1 [10]. Our results indicate that the formation of a quarternary complex of Av_2^r, Av_1 and 2 MgATP cannot be random. At least the second MgATP binding site must be generated after complex formation in the dithionite-reduced $Av_2 \cdot MgATP \cdot Av_1$ complex.

APPENDIX

Scheme 2 gives the species involved during a redox titration of Av_2 in the presence of MgATP. In the presence of MgATP the total Fe protein concentration $[Av_2]_0$ is equal to the sum of the reduced and oxidized forms of Av_2:

$$[Av_2]_0 = [Av_2(red)] + [Av_2(ox)] \quad (5)$$

where $[Av_2(red)] = [Av_2^r] + [Av_2^r \cdot MgATP]$ and

$$[Av_2(ox)] = [Av_2^o] + [Av_2^o \cdot MgATP] + [Av_2^o \cdot (MgATP)_2]. \quad (6)$$

The relation

$$K^r = \frac{[Av_2^r][MgATP]}{[Av_2^r \cdot MgATP]} \quad (7)$$

leads to

$$[Av_2^r \cdot MgATP] = [Av_2^r]c_1 \quad (8)$$

in which K^r is the dissociation constant of $Av_2^r \cdot MgATP$ and

$$c_1 = \frac{[MgATP]}{K^r}.$$

Similarly

$$[Av_2^o \cdot MgATP] = [Av_2^o]c_2, \quad (9)$$

$$[Av_2^o \cdot (MgATP)_2] = [Av_2^o]c_2 c_3 \quad (10)$$

where $c_2 = [MgATP]/K_1^o$, $c_3 = [MgATP]/K_2^o$ and K_1^o and K_2^o are the macroscopic dissociation constants of $Av_2^o \cdot MgATP$ and $Av_2^o \cdot (MgATP)_2$ respectively. Substitution of Eqns (8–10) into Eqns (5) and (6), together with the relation $[Av_2^r] = K[Av_2^o]$, gives:

$$[Av_2]_0 = [Av_2^r \cdot MgATP]\frac{Kc_1 + K + c_2 + c_2 c_3 + 1}{Kc_1}$$

or

$$\frac{[Av_2^r \cdot MgATP]}{[Av_2]_0} = \frac{Kc_1}{K(c_1 + 1) + c_2 + c_2 c_3 + 1}. \quad (11)$$

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REFERENCES

1. Mortenson, L. E. & Thorneley, R. N. F. (1979) *Annu. Rev. Biochem. 48*, 387–418.
2. Thorneley, R. N. F., Eady, R. R. & Lowe, D. J. (1978) *Nature (Lond.) 272*, 557–558.
3. Smith, B. E., Lowe, D. E. & Bray, R. C. (1973) *Biochem. J. 135*, 331–341.
4. Orme-Johnson, W. H. & Davis, L. C. (1977) in *Iron-Sulfur Proteins* (Lovenberg, W., ed.) pp. 15–60, Academic Press, New York.
5. Gillum, W. O., Mortenson, L. E., Chen, J.-S. & Holm, R. H. (1977) *J. Am. Chem. Soc. 99*, 584–595.
6. Stephens, P. J., McKenna, C. E., Smith, B. E., Nguyen, H. T., McKenna, M.-C., Thomson, A. J., Devlin, F. & Jones, J. B. (1979) *Proc. Natl Acad. Sci. USA, 76*, 2585–2589.
7. Braaksma, A., Haaker, H., Grande, H. J. & Veeger, C. (1982) *Eur. J. Biochem. 121*, 483–491.
8. Eady, R. R., Imam, S., Lowe, D. J., Miller, R. W., Smith, B. E. & Thorneley, R. N. F. (1980) in *Nitrogen Fixation* (Stewart, W. D. P. & Gallon, J. R., eds) pp. 19–35, Academic Press, London.
9. Ljones, T. (1979) *FEBS Lett. 98*, 1–8.
10. Hageman, R. V., Orme-Johnson, W. H. & Burris, R. H. (1980) *Biochemistry, 19*, 2333–2342.
11. Eady, R. R., Lowe, D. J. & Thorneley, R. N. F. (1978) *FEBS Lett. 95*, 211–213.
12. Bui, P. T. & Mortenson, L. E. (1969) *Biochemistry, 8*, 2462–2465.
13. Mortenson, L. E., Walker, M. N. & Walker, G. A. (1976) in *Nitrogen Fixation* (Newton, W. E. & Nijman, C. J., eds) pp. 117–149, Washington State University Press, Seattle.
14. Imam, W. & Eady, R. R. (1980) *FEBS Lett. 110*, 35–38.
15. Bui, P. T. & Mortenson, L. E. (1968) *Proc. Natl Acad. Sci. USA, 61*, 1021–1027.
16. Biggens, D. R. & Kelly, M. (1970) *Biochim. Biophys. Acta, 205*, 288–299.
17. Tso, M.-Y. W. & Burris, R. H. (1973) *Biochim. Biophys. Acta, 309*, 263–270.
18. Yates, M. G. (1972) *Eur. J. Biochem. 29*, 386–392.
19. Zumft, W. G., Palmer, G. & Mortenson, L. E. (1973) *Biochim. Biophys. Acta, 292*, 413–421.
20. Miller, R. W., Robson, R. L., Yates, M. G. & Eady, R. R. (1980) *Can. J. Biochem. 58*, 542–548.
21. Ljones, T. & Burris, R. H. (1978) *Biochemistry, 17*, 1866–1872.
22. Eady, R. R. & Smith, B. E. (1978) in *Dinitrogen Fixation* (Hardy, R. W. F., ed.) pp. 399–490, Wiley-Interscience, New York.
23. Thorneley, R. N. F. & Cornish-Bowden, A. (1977) *Biochem. J. 165*, 255–262.
24. Watt, G. D. & Burns, A. (1977) *Biochemistry, 16*, 264–270.
25. Thorneley, R. N. F. (1975) *Biochem. J. 145*, 391–396.
26. Sedmak, J. J. & Grossberg, S. E. (1977) *Anal. Biochem. 79*, 544–552.

27. Kleiner, D. & Chen, C. H. (1974) *Arch. Microbiol. 98*, 93–100.
28. Swisher, R. H., Landt, M. & Reithel, F. J. (1975) *Biochem. Biophys. Res. Commun. 66*, 1476–1482.
29. van Dijk, C., Mayhew, S. G., Grande, H. J. & Veeger, C. (1979) *Eur. J. Biochem. 102*, 317–330.
30. Williamson, J. R. & Corkey, B. E. (1969) *Methods Enzymol. 13*, 481–497.
31. Colowick, S. P. & Womack, F. C. (1969) *J. Biol. Chem. 244*, 774–777.
32. King, E. L. & Altman, C. (1956) *J. Phys. Chem. 60*, 1375–1378.
33. Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660–672.
34. Thorneley, R. N. F. & Eady, R. R. (1973) *Biochem. J. 133*, 405–408.
35. Cermak, V. & Smutek, M. (1975) *Collection Czechoslov. Chem. Commun. 40*, 3241–3264.
36. Klotz, I. M. & Hunston, D. L. (1971) *Biochemistry, 10*, 3065–3069.
37. Zumft, W. G., Mortenson, L. E. & Palmer, G. (1974) *Eur. J. Biochem. 46*, 525–535.

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Chapter 3
Properties of the MgATP and MgADP binding sites on the Fe protein of nitrogenase from Azotobacter vinelandii

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Flow dialysis was used to study the binding of MgATP and MgADP to the nitrogenase proteins of *Azotobacter vinelandii*. Both reduced and oxidized Av_2 bind two molecules of MgADP, with the following dissociation constants: reduced Av_2, $K_1 = 0.091 \pm 0.021$ mM and $K_2 = 0.044 \pm 0.009$ mM; oxidized Av_2, $K_1 = 0.024 \pm 0.015$ mM and $K_2 = 0.039 \pm 0.022$ mM. Binding of MgADP to reduced Av_2 shows positive co-operativity.

Oxidized Av_2 binds two molecules of MgATP with dissociation constants $K_1 = 0.049 \pm 0.016$ mM and $K_2 = 0.18 \pm 0.05$ mM. Binding data of MgATP to reduced Av_2 can be fitted by assuming one binding site, but a better fit was obtained by assuming two binding sites on the protein with negative co-operativity and with dissociation constants $K_1 = 0.22 \pm 0.03$ mM and $K_2 = 1.71 \pm 0.50$ mM.

It was found that results concerning the number of binding sites and the dissociation constants of MgATP-Av_2 and MgADP-Av_2 complexes depend to a great extent on the specific activity of the Av_2 preparation used, and that it is difficult to correct binding data for inactive protein.

No binding of MgADP to Av_1 could be demonstrated. Binding studies of MgADP to a mixture of Av_1 and Av_2 showed that Av_1 did not affect the binding of MgADP to either oxidized or reduced Av_2.

Inhibition studies were performed to investigate the interaction of MgATP and MgADP binding to oxidized and reduced Av_2. All the experimental data can be explained by the minimum hypothesis, i.e. the presence of two adenine nucleotide binding sites on Av_2. MgATP and MgADP compete for these two binding sites on the Fe protein.

Nitrogenase is the enzyme system (reduced carrier: N_2 oxidoreductase ADP-forming) that consists of two dissociating proteins: the larger tetrameric MoFe protein and the smaller dimeric Fe protein. Nitrogenase requires a low-potential electron donor and MgATP for the reduction of N_2 to NH_3. At least two molecules of MgATP are hydrolyzed to MgADP and orthophosphate per electron transferred from the electron donor to the substrates.

The properties of nitrogenase were recently reviewed by Mortenson and Thorneley [1]. Most studies on the mechanism of nitrogenase action have focused on the electron transfer from the artificial electron donor dithionite via the Fe and MoFe proteins to substrates. Relatively little is known about the role of MgATP in nitrogenase catalysis. The interaction of MgATP and MgADP with nitrogenase has been studied by several methods. Information about the role of MgATP in nitrogenase catalysis has been obtained from experiments where binding of MgATP and MgADP to the separated nitrogenase proteins was studied and from experiments where the ATPase activity of the combined nitrogenase proteins was studied.

From the different binding experiments conflicting results have been reported. With column gel filtration Bui and Mortenson [2] demonstrated that Cp_2, but not Cp_1, binds MgATP and MgADP. Biggens and Kelly [3], using the same method of gel filtration, found that both Kp_1 and Kp_2 bind MgATP. The first quantitative results concerning the binding of MgATP and MgADP to the nitrogenase proteins are from Tso and Burris [4]. They found with a gel equilibration method that Cp_2 binds two molecules of MgATP and one molecule of MgADP. Competition studies indicated that MgADP inhibits MgATP binding by occupying one of the two MgATP sites. Later the same laboratory reported that with the gel equilibration method, it was not possible to obtain reproducible binding results with Av_2, mainly caused by the higher dissociation constant of MgATP [5]. Moreover, a reinvestigation of MgATP binding to Cp_2 with the gel equilibration technique indicated 5 ± 2 binding sites [1]. Miller et al. [6] found with the column gel filtration method that Kp_1 binds 4 molecules of MgATP.

Indirect methods used to study the binding of MgATP and MgADP with the Fe protein are EPR spectroscopy [7], circular dichroism [8] and fluorescence titrations [9, 10]. Zumft et al. [7] concluded from their EPR data that Cp_2 binds two molecules of MgATP. Eady and Smith [11], using the same technique for studying the binding of MgATP to Kp_2, criticized the procedure used by Zumft et al. [7] for the inter-

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Abbreviations. The MoFe and Fe proteins of the nitrogenases of *Azotobacter vinelandii*, *Clostridium pasteurianum* and *Klebsiella pneumoniae* are referred to as Av_1 and Av_2, Cp_1 and Cp_2 and Kp_1 and Kp_2, respectively; EPR, electron paramagnetic resonance; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-amino}ethane sulfonic acid; rms, residual mean square; K, stoichiometric or macroscopic dissociation constant; K^i, intrinsic or microscopic dissociation constant.

pretation of the EPR data. McKenna et al. [8, 12] used circular dichroism to study the interaction of MgADP and MgATP with the Fe protein of nitrogenase and found that both oxidized Av_2 and Kp_2 have a high affinity for two molecules of MgADP, and probably also for two molecules of MgATP. You et al. [9, 13] studied the binding of MgATP and MgADP to Av_2 with the aid of the fluorescence probes fluorescein mercuric acetate and fluorescamine. Their data indicate that there are two binding sites for MgATP and at least two for MgADP on Av_2. From Hill plots obtained by fluorescence titrations of the nitrogenase proteins with 1,N^6-etheno-adenosine 5'-triphosphate (εATP) Van Rossen et al. [10] concluded that the ATP analog has one binding site on Av_2 and two binding sites on the complex of Av_1 and Av_2. However, Weston et al. [14] found indications that εATP is hydrolyzed slowly by the nitrogenase complex.

In addition to the direct binding studies, kinetic experiments have also been performed to obtain information about the role of MgATP in nitrogenase catalysis. Ljones and Burris [15] used the reaction between the Fe protein and bathophenanthroline disulfonate as a probe for the interactions with MgATP and MgADP. They concluded that the data obtained with this indirect method were consistent with the model given by Tso and Burris [4] for binding of MgATP and MgADP to Cp_2. Sigmoidal dependence of the reaction rate of bathophenanthroline disulfonate with reduced Av_2 upon concentration of MgATP was used as a prove for binding of two molecules of MgATP per molecule Av_2. However, we showed that there is also another interpretation possible of these kinetic data, with only one MgATP binding site present on Av_2 [16]. From steady-state and pre-steady-state kinetic data information was obtained about the role of MgATP in nitrogenase catalysis. With stopped-flow experiments Hageman et al. [5] demonstrated that there is a sigmoidal dependence of the rate of electron transfer between the nitrogenase proteins on the MgATP concentration. Their data indicated that the electron transfer reaction is dependent on the presence of two MgATP molecules with K_m values of 0.97 mM and 0.22 mM. From pre-steady-state kinetics Thorneley and Cornish-Bowden [17] concluded that MgADP is a competitive inhibitor of MgATP in the MgATP-induced electron transfer from Kp_2 to Kp_1. However, they observed a heterotrophic interaction between MgATP and MgADP binding to nitrogenase in kinetic assays of H_2 evolution.

It may be clear from the studies cited above that the technique used to study the interaction of MgATP and/or MgADP with the nitrogenase proteins determines to a great extent the results obtained. One possible reason for the discrepancies in binding results is the variation in the activities of the Fe proteins used in the different experiments. In the present study the effect of the activity of Av_2 on the MgATP binding properties of the oxidized and reduced protein has been investigated. This paper is an extension of our earlier work on the binding of MgATP to Av_2. Here we present flow dialysis data of the binding of MgATP and MgADP to the different oxidation states of Av_2, and of the interactions between MgATP and MgADP binding. From these results, a model for MgATP and MgADP binding to Av_2 is postulated.

MATERIALS AND METHODS

Cell growth and enzyme preparation

Growth conditions of *Azotobacter vinelandii* ATCC strain 478 and the isolation of the nitrogenase proteins were the same as described earlier [16]. The purified preparations of Av_1 and Av_2 had specific activities of about 2500 and 2000 nmol ethylene produced · min^{-1} · (mg protein)$^{-1}$, respectively. During a last purification step fractions were also obtained which are pure on gel but have a lower specific activity. These fractions were used when protein with a low specific activity was tested. Phenazine methosulfate was used to oxidize Av_2. A Bio-Gel P-6DG column (1 cm × 10 cm) equilibrated with anaerobic 50 mM Tes/NaOH buffer containing 5 mM $MgCl_2$ (final pH 7.4) was loaded with 0.3 ml 20 mM phenazine methosulfate. Oxidation of Av_2 was performed by running 0.3 ml concentrated Av_2 solution through the layer of phenazine methosulfate and subsequent desalting.

Analytical methods

Nitrogenase activity was measured at pH 7.4 and 30 °C under the standard assay conditions described by Braaksma et al. [18], with optimal levels of the complementary protein. The concentrations of the nitrogenase proteins were calculated on the basis of relative molecular mass values of 222 kDa and 63 kDa for Av_1 and Av_2, respectively. Protein concentrations were estimated by the microbiuret method of Goa [19] and/or the Coomassie brilliant blue method of Sedmak and Grossberg [20] calibrated with bovine serum albumin. Braaksma et al. [21] found that the protein determination of purified Av_2 by the method of Sedmak and Grossberg [20] gives an underestimation of 25–30% compared to the biuret or Lowry method and dry weight determinations.

Radiochemical purity of [2,8-^{3}H]ATP and [2,8-^{3}H]ADP were checked prior to use by chromatography on poly(ethyleneimine)-cellulose thin-layer sheets (Merck) using 2M LiCl as the developing solvent. Non-radioactive MgATP and MgADP were cochromatographed with the radioactive samples to locate the position of the nucleotides by fluorescence-quenching on irradiation with ultraviolet light. Radioactive spots were detected by autoradiography using X-Omat-AR-5 film (Kodak). R_F values of MgATP and MgADP were 0.76 and 0.85, respectively. A radiochemical impurity generated during storage of the radioactive nucleotides gave a decomposition product with an R_F value of 0.21.

The flow dialysis technique developed by Colowick and Womack [22] was used to study the binding of MgATP and MgADP to the nitrogenase proteins. The flow dialysis experiments were performed at room temperature (20–22 °C) as previously described [16], with (2,8-^{3}H)-labeled ATP or ADP as radioactive ligand. Gel filtration on Sephacryl S-200 was used to prepare stock solutions of reduced Av_2 (about 12 mg/ml) in 50 mM Tris/HCl buffer, containing 5 mM $MgCl_2$ and 2 mM $Na_2S_2O_4$ (final pH 7.4). The stock solution was used directly for flow dialysis experiments and was diluted only by the addition of concentrated adenine nucleotides.

Oxidized Av_2 was prepared as described above using a Bio-Gel P-6DG column equilibrated with anaerobic 50 mM Tes/NaOH buffer containing 5 mM $MgCl_2$ (final pH 7.4). This protein stock solution (about 12 mg/ml) was used directly for flow dialysis experiments.

The oxidation state and activity of the protein before and after the flow dialysis experiments were checked. Only in the case of oxidized Av_2 in the presence of high MgATP concentrations there was a slight decrease in activity (about 20%).

Binding experiments of MgATP and MgADP with Av_2 by gel filtration were performed according to the method of Hummel and Dryer [23]. A column (1 cm × 17 cm) containing Bio-Gel P-6DG was equilibrated with anaerobic buffer containing 50 mM Tes/NaOH, 5 mM $MgCl_2$ and 1 mM MgATP or MgADP (final pH 7.4). When binding to reduced Av_2 was measured 2 mM $Na_2S_2O_4$ was added to the equilibration buffer. Binding measurements to oxidized Av_2 were carried out by first loading the equilibrated column with 0.3 ml 20 mM phenazine methosulfate, followed by chromatography of 0.3 ml of concentrated Av_2. For binding studies to reduced Av_2 the column was loaded directly with 0.3 ml of the concentrated Fe protein (±40 mg/ml). The elution rate was about 0.3 ml/min. The effluent solution was collected in 0.5-ml fractions for further analysis. Protein-containing fractions were collected anaerobically and aliquots were injected immediately into 6% perchloric acid for measurement of the nucleotide concentration. ATP and ADP concentrations were determined enzymatically as described by Williamson and Corkey [24].

Fitting of models

Non-linear models were fitted to the binding data using the statistical program package BMDP, release 1981, program 3R (University of California). This program performs the method of least-squares to fit the data to the appropriate equation.

Chemicals and gases

All chemicals employed were of the purest grade available and were obtained from commercial sources. [2,8-^{3}H]ATP and [2,8-^{3}H] ADP, sodium salts (specific activity ≈30 Ci/mmol) were purchased from New England Nuclear. Argon gas was freed from oxygen by passing it over a BASF catalyst at about 120°C.

RESULTS

Binding of MgATP to Av_2

An important factor in all studies with nitrogenase proteins is the lability of the proteins. Published work on nitrogenase shows a considerable variation in the specific activities of the component proteins. Both the MoFe and the Fe protein are O_2-sensitive, the latter protein to the greater extent. The presence of substantial proportions of O_2-inactivated protein and/or apoprotein introduces ambiguity in the interpretation of results obtained from studies with nitrogenase. It is not uncommon that experimental results are corrected by extrapolation to fully active protein. Hageman et al. [5] reported that during the pre-steady-state burst of MgATP hydrolysis, 1.3 molecules of MgATP are hydrolyzed per molecule of Av_2. They assumed that under the experimental conditions used only an Av_2 molecule containing a [4Fe-4S] cluster hydrolyses MgATP. The concentration of active Av_2 was taken to be the concentration of the [4Fe-4S] clusters in the protein, as estimated by the ATP-sensitive bathophenanthroline Fe chelation assay of Ljones and Burris [15]. Using Av_2 with a specific activity of 1750 nmol C_2H_2 reduced · min^{-1} · mg^{-1} and 0.53 [4Fe-4S] cluster, they corrected the value of 1.3 to 2.5 MgATP molecules hydrolyzed per electron transferred from Av_2 to Av_1. From circular dichroic studies in which oxidized Av_2 was titrated with MgADP, McKenna et al. [8] concluded that there are two MgADP sites on Av_2. This value was obtained after correcting for the presence of inactive protein in the sample, using an Av_2 preparation with a specific activity of 1750 nmol C_2H_2 reduced · min^{-1} · mg^{-1} and 0.58 [4Fe-4S] cluster per protein molecule. However, if inactive Av_2 also binds MgADP, such a correction of the titration is not allowed.

Aside from the question whether the method used for calculation of the percentage inactive protein in a given preparation is correct, one can ask whether it is always realistic to correct experimental data for inactive protein. Correction using the ATP-sensitive bathophenanthroline Fe chelation assay [15] might be important for electron transfer properties of the Fe protein, but it is not known whether this is also correct for the adenine nucleotide binding properties of the Fe protein. To obtain an idea of the effect of the specific activity of Av_2 on the binding properties of the protein, binding experiments were carried out using Av_2 preparations with different specific activities. By plotting the binding data according to Scatchard [25], the two parameters that characterize the binding equilibria are obtained, namely the number of binding sites N and the dissociation constants K_d. It is shown in Table 1 that there is a significant difference in the values of the binding parameters (K_d and N) obtained from different protein preparations. In some cases the differences are well outside the standard deviation of a binding experiment. This is not observed when protein preparations are used with a specific activity above 2000 nmol C_2H_4 formed · min^{-1} · mg Av_2^{-1}. For five different protein preparations with a specific activity above 2000 nmol C_2H_4 formed · min^{-1} · mg Av_2^{-1}, the binding parameters were calculated according to Scatchard [25] (not shown). All values are situated between the standard deviations given in Table 1 for the protein preparations with specific activities above 2000 nmol C_2H_4 formed · min^{-1} · mg Av_2^{-1}. In a previous study [16], we reported binding of MgATP to Av_2 with a specific activity of 2200 nmol C_2H_2 reduced · min^{-1} · mg^{-1}. During that study the protein concentration was estimated by the Coomassie brilliant blue method [20] with bovine serum albumin as standard. As pointed out under Materials and Methods, the Coomassie brilliant blue method gives an underestimation of the protein concentration of 25–30% compared to the biuret or the Lowry methods [21]. The recalculated values for the specific activity of this Av_2 preparation and the number of MgATP binding sites, obtained after correction for protein concentrations, are given in Table 1.

The binding parameters of Table 1 were obtained by analysis according to Scatchard [25] of flow dialysis data. For a protein with only one binding site for the ligand, a Scatchard plot is a convenient and reliable method for the calculation of the binding constant. In the case of more binding sites, the simplest situation is that all binding sites are identical and have the same affinity for the ligand. Only for these two situations will the Scatchard graph be linear. So, in many cases, a Scatchard plot proves to be curved rather than linear. From the Scatchard plot representing MgATP binding to reduced Av_2 with a specific activity of 2335 nmol C_2H_2 reduced · min^{-1} · mg^{-1} a value of N of 1.6 was obtained (see Table 1), so it is not clear whether there are one or two MgATP binding sites.

To obtain information on whether there is deviation from a straight line in a Scatchard plot, it is important to have binding data over a wide range of ligand concentrations. Therefore more flow dialysis experiments were carried out using several Av_2 preparations, all with a high specific activity

Table 1. *Effect of the specific activity of Av_2 on the binding of MgATP as determined by flow dialysis*
Binding of MgATP was measured to Av_2 preparations with different specific activities. Oxidation of Av_2 was performed with phenazine methosulfate (or sulfite, see footnote). The binding data were plotted on a Scatchard graph and fitted to a straight line. A linear regression analysis was employed to obtain the values for the number of binding sites (N) and the dissociation constants (K_d)

Oxidation state of Av_2	Specific activity	K_d	N
	nmol · min^{-1} · mg^{-1}	mM	
Reduced	2335	0.42 ± 0.08	1.6 ± 0.3
	1725[a]	0.56 ± 0.11[a]	1.0 ± 0.2[a]
	1650	0.80 ± 0.14	1.1 ± 0.2
	1608	0.45 ± 0.07	1.2 ± 0.2
Oxidized	2120	0.14 ± 0.02	2.2 ± 0.2
	1922	0.18 ± 0.05	1.7 ± 0.4
	1725[a]	0.29 ± 0.05[a]	1.7 ± 0.3[a]
	980	0.09 ± 0.02	2.8 ± 0.5
	557	0.15 ± 0.06	2.1 ± 0.5

[a] These are the recalculated values of binding data published previously [16] in which Av_2 was oxidized with sulfite.

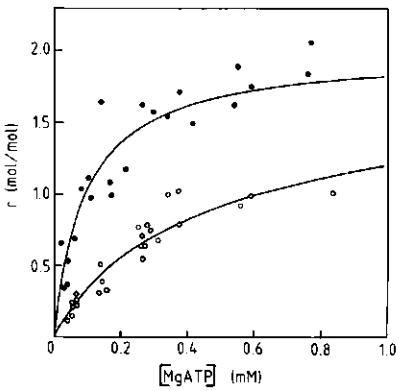


Fig. 1. *Binding of [2,8-^{3}H]MgATP to reduced and oxidized Av_2 as determined by flow dialysis.* Flow dialysis experiments were carried out as described in Materials and Methods. Av_2 preparations used had a specific activity of at least 2000 nmol C_2H_2 reduced · min^{-1} · mg^{-1}. Av_2 was oxidized with phenazine methosulfate as detailed in Materials and Methods. The curves drawn through the data points are computer fits according to Eqn (2) as described in Results. r = mol MgATP bound/mol Av_2. (○——○), Reduced Av_2; (●——●) oxidized Av_2

(> 2000 nmol C_2H_2 reduced · min^{-1} · mg^{-1}). Fig. 1 is constructed from flow dialysis experiments performed at 26 different MgATP concentrations in the case of reduced Av_2 (five different protein batches) and at 22 different MgATP concentrations in the case of oxidized Av_2 (five different protein batches). Instead of a graphical analysis of the binding data according to Scatchard, we used computer-fitting techniques for the determination of the binding parameters of Figs 1–4. Such a method allows the quantitative fitting of saturation curves and the determination of standard errors of the estimated parameters.

The saturation curve for binding of a ligand to a protein which has only one binding site is expressed by the following equation:

$$r = \frac{[ATP]/K}{1 + [ATP]/K} \quad (1)$$

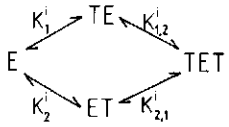
in which r is the ratio of the concentration of bound ligand to the total concentration of protein and K is the dissociation constant. When one assumes one binding site for MgATP on reduced Av_2, the value of K which gives the best fit of Eqn (1) to the experimental data of Fig. 1 is 0.15 ± 0.02 mM (rms = 0.015). However, graphical analysis of the MgATP binding data obtained with reduced Av_2 of high specific activity (Table 1) indicates more than one binding site for MgATP on the protein ($N = 1.6$). To find out whether there is a second binding site for MgATP on reduced Av_2 with lower affinity, one needs binding data at high concentrations of MgATP. But binding data above 1 mM MgATP are difficult to obtain because with flow dialysis, the fraction of MgATP bound to protein is measured with respect to the free concentration of MgATP. This means that the accuracy of the results becomes smaller when the concentration of free ligand increases. Also an increase in protein concentration is, for practical reasons, not possible. This is caused by the very low ATPase activity of Av_2 preparations (< 1 nmol ATP hydrolyzed · min^{-1} · mg Av_2^{-1}). At very high protein concentrations and at saturating ATP concentrations, the ATPase activity becomes significant during the time course of a flow dialysis experiment (10 min are necessary to perform a measurement) and the ADP formed influences the ATP binding significantly. This is not the case at protein concentrations below 20 mg/ml.

When one assumes two binding sites for MgATP on reduced Av_2, then the number of molecules of MgATP bound per molecule of protein can be expressed in the form of Eqn (2):

$$r = \frac{[ATP]/K_1 + 2[ATP]^2/K_1K_2}{1 + [ATP]/K_1 + [ATP]^2/K_1K_2}. \quad (2)$$

In this equation the constants K_1 and K_2 represent the stoichiometric or macroscopic dissociation constants. When the MgATP binding data for reduced Av_2 were fitted with Eqn (2), the values obtained for K_1 and K_2 were 0.22 ± 0.03 mM and 1.71 ± 0.50 mM, respectively (rms = 0.009) and thus a better fit to the binding data of Fig. 1 than with Eqn (1). Thus one has to consider seriously the possibility of two MgATP binding sites on reduced Av_2. Oxidation of reduced Av_2 results in two binding sites for MgATP on the Fe protein (Fig. 1). If there were only one binding site for MgATP on reduced Av_2, oxidation of reduced Av_2 means the generation of a second MgATP binding site. It seems more logical that upon oxidation of Av_2 the number of binding sites remains the same but that the affinity of the low-affinity site for MgATP increases.

The stoichiometric dissociation constants K_1 and K_2 given above for the binding of MgATP to reduced Av_2 describe only the binding behaviour of the whole protein and give no information about the individual sites. By using the relation between the stoichiometric binding constants and the intrinsic or microscopic binding constants, in some cases information can be obtained about the molecular properties of the individual binding sites.



Scheme 1. *Intrinsic dissociation constants for the binding of a ligand (T) to a protein (E) with two binding sites*

The relation between the stoichiometric (K_1 and K_2) and intrinsic (K_1^i, K_2^i, $K_{1,2}^i$ and $K_{2,1}^i$) dissociation constants in a two-site system is given by the following equations [26]:

$$(K_1)^{-1} = (K_1^i)^{-1} + (K_2^i)^{-1} \quad (3)$$

and

$$(K_1 K_2)^{-1} = (K_1^i K_{1,2}^i)^{-1}. \quad (4)$$

When the intrinsic dissociation constants are identical ($K_1^i = K_2^i = K_{1,2}^i = K_{2,1}^i$), the stoichiometric dissociation constants differ by a factor 4 ($K_1 = 0.25\ K_2$). The observed values of the stoichiometric dissociation constants for MgATP binding to reduced Av_2 are 0.22 mM and 1.71 mM, differing by more than a factor 4 (t-test: $P = 0.18$). When it is assumed that the intrinsic dissociation constants for MgATP binding to reduced Av_2 are not identical, then there are two distinct situations in which the values of the intrinsic dissociation constants can still be determined from the stoichiometric dissociation constants using Eqns (3) and (4). In the first case, if the two sites are non-interacting and have different affinities for ligand, then $K_1^i = K_{2,1}^i$ and $K_2^i = K_{1,2}^i$. This leads to values for the intrinsic dissociation constants K_1^i and K_2^i of 0.25 ± 0.06 mM and 1.45 ± 0.55 mM, respectively.

The other case that allows determination of the intrinsic dissociation constants is that in which the sites are initially identical and the second molecule binds with negative or positive co-operativity, so that $K_1^i = K_2^i$ and $K_{2,1}^i = K_{1,2}^i$. By using Eqns (3) and (4), it follows that $K_1 = 0.5\ K_1^i$ and $K_2 = 2\ K_{1,2}^i$. For the case of MgATP binding to reduced Av_2, $K_1^i = 0.44 \pm 0.06$ mM and $K_{1,2}^i = 0.85 \pm 0.25$ mM. In all other alternative binding patterns it is not possible to obtain information from the stoichiometric binding constants about the intrinsic binding constants.

Independent evidence is required to determine whether the two sites on reduced Av_2 are initially the same or have intrinsically different affinities for MgATP. However, in view of what is known of the structure of Av_2, namely that it is a dimer of two identical subunits, it seems reasonable to assume that both sites in the original non-ligand protein are identical. Additionally, there are indications that the conformation of reduced Av_2 is changed on binding MgATP, since (a) its [4Fe-4S) cluster reacts with 2,2'-bipyridyl [27] and bathophenanthroline disulfonate [5] after adding MgATP, and (b) its O_2-sensitivity increases as well as the number of -SH groups reacting with 5,5'-dithiobis(2-nitrobenzoate) [28] in the presence of MgATP.

It is not clear yet whether all these changes are induced upon binding of either one or two molecules of MgATP to the Fe protein. But, if binding of the first MgATP molecule to Av_2 causes a conformation change of the protein, then this may result in a lower affinity for the second MgATP.

Oxidized Av_2 binds two molecules of MgATP (Fig. 1). When the flow dialysis data for the binding of MgATP to oxidized Av_2 were fitted with Eqn (2), the best fit was obtained with the stoichiometric dissociation constants $K_1 = 0.049 \pm 0.016$ mM and $K_2 = 0.18 \pm 0.05$ mM (rms = 0.037). Since

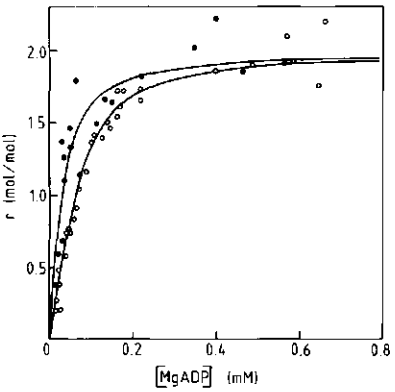


Fig. 2. *Binding of [2,8-^{3}H]MgADP to reduced and oxidized Av_2 as determined by flow dialysis.* (○——○). Reduced Av_2; (●——●) oxidized Av_2

the stoichiometric binding constants K_1 and K_2 differ by a factor of almost 4 (t-test: $P = 0.87$), this indicates that the intrinsic dissociation constants for the binding of MgATP to oxidized Av_2 have about the same value, $K^i = 0.095 \pm 0.029$ mM. We can conclude from the curve-fitting analysis of the data in Fig. 1 that oxidized Av_2 and reduced Av_2 have two binding sites for MgATP. Oxidized Av_2 has a 5–9 times higher affinity for MgATP than reduced Av_2, while the binding to reduced Av_2 shows a negative co-operative behaviour.

Binding of MgADP to Av_2

Our previous work on the binding of MgATP to Av_2 [16] has shown that the Fe protein is not very stable under most of the experimental conditions used for binding studies. We therefore examined the stability of Av_2 in the presence of MgADP. Neither reduced nor phenazine-methosulfate-oxidized Av_2 was inactivated in the presence of 1 mM MgADP during anaerobic incubation for 2 h. For the MgATP binding studies described earlier [16], sulfite-oxidized Av_2 was used. When phenazine-methosulfate-oxidized Av_2 was incubated with 1 mM MgATP, we observed inactivation of the protein with a half-time of more than 2 h, indicating that phenazine-methosulfate-oxidized Av_2 is more stable in the presence of MgATP than is sulfite-oxidized Av_2 [16].

Fig. 2 shows the results obtained by the flow dialysis technique for the binding of MgADP to reduced and oxidized Av_2. At a free MgADP concentration around 0.8 mM, both oxidation states of the Fe protein bind two molecules of MgADP. Estimation of the stoichiometric dissociation constants as given in Eqn (2) using the binding data of Fig. 2 gives for reduced Av_2 $K_1 = 0.091 \pm 0.021$ mM and $K_2 = 0.044 \pm 0.009$ mM (rms = 0.011) and for oxidized Av_2, $K_1 = 0.024 \pm 0.015$ mM and $K_2 = 0.039 \pm 0.022$ mM (rms = 0.067). Since in the case of binding of MgADP to reduced Av_2 $K_1 > 0.25\ K_2$, this indicates substantial positive co-operativity, i.e. $K_1^i \neq K_{2,1}^i$ and $K_2^i \neq 2K_{1,2}^i$ (see Scheme 1). The only situation that allows calculation of the intrinsic dissociation constants from Eqns (3) and (4) is if $K_1^i = K_2^i$ and $K_{2,1}^i = K_{1,2}^i$. The calculated values for the intrinsic dissociation constants of MgADP binding to reduced Av_2 then are: K_1^i

$= 0.18 \pm 0.04$ mM and $K^i_{1,2} = 0.022 \pm 0.005$ mM. When a similar assumption is made for oxidized Av_2 then the calculated values are $K^i_1 = 0.048 \pm 0.030$ mM and $K^i_{1,2} = 0.019 \pm 0.011$ mM. While for reduced Av_2 the intrinsic dissociation constants for MgADP binding differ by a factor 8, a difference that was found to be significant (t-test: $P = 0.002$), this difference for oxidized Av_2 is only by a factor of 2.5 (t-test: $P = 0.51$). Therefore, one can conclude that: firstly, oxidized Av_2 binds MgADP 2–3-fold stronger than reduced Av_2; secondly, the binding of MgADP to reduced Av_2 shows positive co-operative behaviour; thirdly, oxidized Av_2 binds both MgATP and MgADP more strongly than reduced Av_2; and fourthly, MgADP has a higher affinity for both reduced and oxidized Av_2 than does MgATP.

Preliminary results published recently [29] on the binding of MgADP to Av_2 indicated only one binding site for MgADP to both reduced and oxidized Av_2. These preliminary binding curves seem to flatten at a saturation degree of around 0.7 mol MgADP bound/mol Av_2. It was found later that these low binding numbers were due to impurities with an R_F value of 0.21 in the [^{3}H]MgADP used for the flow dialysis experiments (see Materials and Methods). This impurity resulted in a substantial background of radioactivity in the fractions collected during a flow dialysis experiment, thereby disturbing the calculation of free and bound MgADP. The batches of [^{3}H]MgATP and [^{3}H]MgADP used in the present experiments were more than 95% pure as analysed by thin-layer chromatography.

The binding data obtained by flow dialysis were checked with an independent method, namely column gel filtration (see Materials and Methods). In these gel filtration experiments the amount of nucleotide bound to Av_2 was measured enzymatically. With the gel filtration method we found that at a free MgATP concentration around 1 mM, reduced Av_2 binds about one molecule of MgATP, a value expected from the stoichiometric dissociation constants, and that oxidized Av_2 binds two molecules of MgATP (data not shown). In addition the results obtained by flow dialysis with respect to the number of MgADP binding sites present on reduced and oxidized Av_2 were confirmed by gel filtration experiments.

Inhibition studies of MgATP and MgADP binding to Av_2

Flow dialysis experiments were carried out to investigate whether MgATP binding to Av_2 is influenced by MgADP and vice versa. In the competition experiments unlabeled MgADP was used as inhibitor of the binding of [^{3}H]MgATP, while unlabeled MgATP was used as inhibitor of [^{3}H]MgADP binding. When the protein concentration in a binding experiment is small with respect to the inhibitor concentration, the total concentration of inhibitor is almost equal to the concentration of free inhibitor. In all our experiments the protein concentration is around 0.2 mM, so corrections had to be made, especially at the lower inhibitor concentrations.

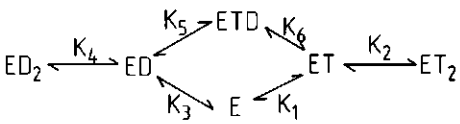
All data points with equal concentrations of added MgATP and MgADP were compared. When radioactive MgATP was used the ratio of free to bound MgATP was calculated. From an identical experiment, but with MgADP as the radioactive ligand, the ratio of free to bound MgADP was calculated. These results were used to construct the competition curves as shown in Figs 3 A, B and 4 A, B. Because the binding of MgATP influences the binding of MgADP and vice versa, during a titration with MgADP at a constant total concentration of the inhibitor MgATP, the ratio of free to bound MgATP will differ at the different MgADP concentrations. This is the reason for the range of free inhibitor concentrations given in the legends of Figs 3 and 4.

The inhibition patterns (drawn lines) as shown in Figs 3 and 4 are computed curves obtained for the free MgADP and MgATP concentrations as indicated. Fig. 3 A shows that there is a strong inhibition of MgATP binding to reduced Av_2 when MgADP is added. As shown in Fig. 3 B, the inhibitory effect of MgATP on the binding of MgADP to reduced Av_2 is much smaller. In the same way as done for reduced Av_2, competition experiments were performed with oxidized Av_2 (Figs 4 A, B). Again, there is a strong inhibition of MgATP binding to oxidized Av_2 by MgADP, and a smaller effect of MgATP on the binding of MgADP.

From the binding experiments described in Figs 1 and 2 it is clear that both oxidized and reduced Av_2 have two binding sites for MgATP and also two for MgADP. Therefore three different possibilities can be visualized for simultaneous binding of MgATP and MgADP to the Fe protein.

Firstly, there are four binding sites present on Av_2, two for MgATP and two for MgADP, which means interaction between the binding sites of MgATP and MgADP, resulting in a lower affinity for MgADP when MgATP has already bound to the protein, and vice versa. Secondly, there are three adenine nucleotide binding sites present on Av_2, one of which can bind both MgATP and MgADP. Thirdly, there are only two adenine nucleotide binding sites present on Av_2, which means competition between MgATP and MgADP for the same sites. Because the adenine nucleotide which has the highest affinity for Av_2 (MgADP) shows the strongest inhibition, the simplest explanation is competition of MgADP and MgATP for the same two sites. Moreover, it can be calculated from the data points presented in Figs 3 A, B and 4 A, B that the total amount of MgATP plus MgADP bound simultaneously to Av_2 never exceeds two per molecule of Av_2. To check the flow dialysis data gel filtration binding experiments were performed with different ratios of MgATP and MgADP. These experiments also indicated that the total amount of the two adenine nucleotides bound to Av_2 never exceeds two per molecule of protein (data not shown). For these reasons, the binding data of Figs 3 and 4 were tested to see if they can be explained by the simplest model, i.e. the two-site system schematically represented by Scheme 2.

In Scheme 2 it is formulated that Av_2 has two adenine nucleotide binding sites which can bind either MgATP or MgADP.



Scheme 2. *Stoichiometric dissociation constants for the binding of MgATP (T) and MgADP (D) to the Fe protein (E)*

In the presence of both MgATP and MgADP, the ratio r, of the concentration of bound MgATP to the total concentration of Fe protein is given by the following equation:

$$r = \frac{[ATP]/K_1 + 2[ATP]^2/K_1K_2 + [ATP][ADP]/K_1K_6}{1 + [ATP]/K_1 + [ADP]/K_3 + [ATP]^2/K_1K_2 + [ADP]^2/K_3K_4 + [ATP][ADP]/K_1K_6}. \quad (5)$$

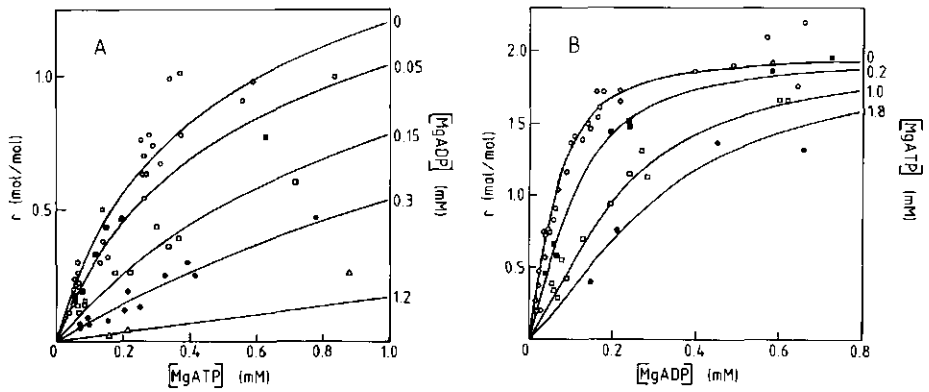


Fig. 3. *Binding of [2,8-^{3}H]MgATP and [2,8-^{3}H]MgADP to reduced Av_2 in the presence of variable concentrations of unlabeled MgADP and unlabeled MgATP, respectively.* Data were obtained from flow dialysis experiments (see Materials and Methods and also the legend of Fig. 1). The concentration of free inhibitor was calculated as described in Results and Discussion. The curves drawn through the data points are computer fits according to Eqn (5) for the free inhibitor concentrations of MgADP (A) and MgATP (B) as indicated. (A) Binding of [2,8-^{3}H]MgATP to reduced Av_2 in the absence (○——○) and presence of variable concentrations of free MgADP: (■——■) 0.05 – 0.1 mM; (□——□) 0.1 – 0.2 mM; (●——●) 0.2 – 0.5 mM; (△——△) 1.0 – 1.5 mM. r = mol MgATP bound/mol Av_2. (B) Binding of [2,8-^{3}H]MgADP to reduced Av_2 in the absence (○——○) and presence of variable concentrations of free MgATP: (■——■) 0.15 – 0.25 mM; (□——□) 0.8 – 1.2 mM; (●——●) 1.7 – 2.0 mM. r = mol MgADP bound/mol Av_2

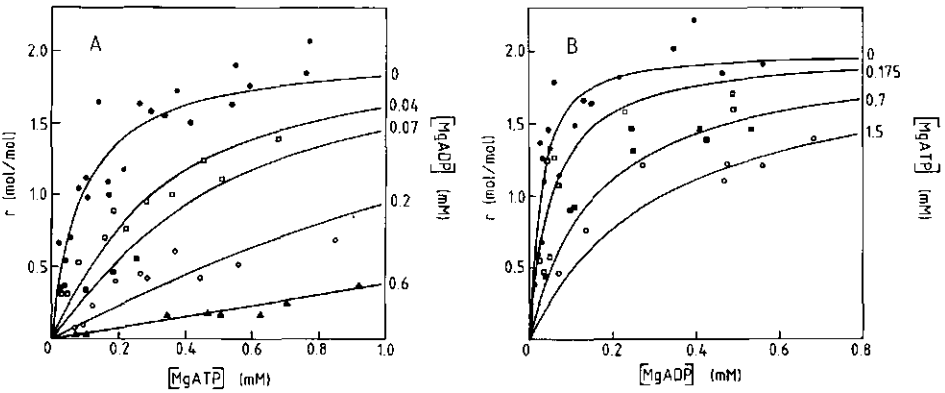


Fig. 4. *Binding of [2,8-^{3}H]MgATP and [2,8-^{3}H]MgADP to oxidized Av_2 in the presence of variable concentrations of unlabeled MgADP and unlabeled MgATP, respectively.* Experimental conditions as described in the legend of Fig. 3. (A) Binding of [2,8-^{3}H]MgATP to oxidized Av_2 in the absence (●——●) and presence of variable concentrations of free MgADP: (□——□) 0.03 – 0.05 mM; (■——■) 0.06 – 0.08 mM; (○——○) 0.15 – 0.25 mM; (▲——▲) 0.4 – 0.7 mM. r = mol MgATP bound/mol Av_2. (B) Binding of [2,8-^{3}H]MgADP to oxidized Av_2 in the absence (●——●) and presence of variable concentrations of free MgATP: (□——□) 0.15 – 0.25 mM; (■——■) 0.7 – 1.0 mM; (○——○) 1.4 – 1.7 mM. r = mol MgADP bound/mol Av_2

Using Eqn (5) it is possible to compute the saturation curves for the binding of MgATP to reduced and oxidized Av_2 in the presence of different concentrations of free MgADP. Fig. 3A shows the computed curves using values for the stoichiometric dissociation constants of MgATP and MgADP to reduced Av_2 as estimated from the curves in Figs 1 and 2, namely $K_1 = 0.22$ mM, $K_2 = 1.71$ mM, $K_3 = 0.091$ mM and $K_4 = 0.044$ mM. Non-linear regression gives as best fit for the dissociation constant K_6 a value of 0.13 ± 0.02 mM (rms = 0.002). Using the relation

$$K_1 K_6 = K_3 K_5 \qquad (6)$$

the value of $K_5 = 0.31 \pm 0.03$ mM can be obtained. The same kind of relation as given by Eqn (5) can be derived for the binding of MgADP to Av_2 in the presence of MgATP. Fig 3B shows the computed curves for the binding of [^{3}H]MgADP to reduced Av_2 in the presence of different concentrations of MgATP. The best fit to the binding data was obtained using a value for K_5 of 0.40 ± 0.14 mM (rms = 0.008). Eqn (6) then gives as value for $K_6 = 0.17 \pm 0.06$ mM.

The stoichiometric dissociation constants K_5 and K_6 are equal to the intrinsic dissociation constants K_5^i and K_6^i, respectively. Table 2 shows the values for K_5^i and K_6^i obtained by computer fitting of the binding data of Figs 3A, B. The fitted values for K_5^i and K_6^i obtained from the MgATP binding experiments of Fig. 3A agree within the experimental error with those obtained from the MgADP binding experiments of Fig. 3B.

Table 2. *Intrinsic dissociation constants for the binding of MgATP and MgADP to reduced and oxidized Av_2*
$K_1^i - K_6^i$ are the intrinsic dissociation constants for the binding of MgATP and MgADP to Av_2 as defined in Schemes 1 and 2. For the calculation of K_1^i, $K_{1,2}^i$, K_3^i and $K_{3,4}^i$ from the binding data of Figs 1 and 2 it was assumed that initially the two binding sites for MgATP, and also those for MgADP, are identical and have the same affinity for ligand. For the calculation of K_5^i and K_6^i from the binding data of Figs 3 and 4 it was assumed that MgATP and MgADP have the same binding sites on Av_2

Oxidation state of Av_2	Dissociation constant for			
	MgATP-binding	MgADP-binding	MgATP-binding (competition)	MgADP-binding (competition)
	mM			
Reduced	$K_1^i = 0.44 \pm 0.06$ $K_{1,2}^i = 0.85 \pm 0.25$ (rms = 0.009)	$K_3^i = 0.18 \pm 0.04$ $K_{3,4}^i = 0.022 \pm 0.005$ (rms = 0.011)	$K_5^i = 0.31 \pm 0.03$ $K_6^i = 0.13 \pm 0.02$ (rms = 0.002)	$K_5^i = 0.40 \pm 0.14$ $K_6^i = 0.17 \pm 0.06$ (rms = 0.008)
Oxidized	$K_1^i = 0.095 \pm 0.029$ $K_{1,2}^i = 0.095 \pm 0.029$ (rms = 0.037)	$K_3^i = 0.048 \pm 0.030$ $K_{3,4}^i = 0.019 \pm 0.011$ (rms = 0.067)	$K_5^i = 0.075 \pm 0.018$ $K_6^i = 0.038 \pm 0.009$ (rms = 0.013)	$K_5^i = 0.059 \pm 0.016$ $K_6^i = 0.030 \pm 0.008$ (rms = 0.032)

As discussed for the binding experiments with MgATP and MgADP alone, with a two-site model there are only two situations in which the values of the intrinsic binding constants can be obtained directly from the stoichiometric binding constants. In one instance the two sites are non-interacting, but have different affinities for ligand. Since MgADP binding to reduced Av_2 shows positive co-operativity, it was concluded that the binding sites for MgADP, and thus also for MgATP, are initially identical.

Table 2 summarizes the values obtained for the different intrinsic dissociation constants using the relations from Scheme 1: $K_1 = 0.5\,K_1^i$, $K_2 = 2K_{1,2}^i$, $K_3 = 0.5\,K_3^i$, $K_4 = 2K_{3,4}^i$. It is clear that the values of K_3^i and K_6^i are identical within experimental error. This means that, although binding of MgATP to one site of reduced Fe protein lowers the affinity of the second binding site for MgATP, it has no appreciable effect on the affinity for MgADP. For the same reason ($K_1^i \approx K_5^i$) MgADP binding to one site of reduced Av_2 has no effect on the binding constant of MgATP for the second site.

In the same way as described for reduced Av_2, the binding data obtained for the simultaneous binding of MgATP and MgADP to oxidized Av_2 can be fitted with Eqn (5). The computed inhibition patterns are shown in Figs 4A and 4B. The dissociation constants for the binding of MgATP and MgADP to oxidized Av_2 used in computing the curves of Fig. 4 are those determined from the binding curves in Fig. 1 and 2, namely $K_1 = 0.049$ mM, $K_2 = 0.18$ mM, $K_3 = 0.24$ mM and $K_4 = 0.039$ mM. Table 2 shows that the values of K_5^i and K_6^i obtained from each set of competition experiments, as represented by Figs 4A and 4B, do not differ much from each other. Assuming that the adenine nucleotide binding sites are initially the same, it is clear from Table 2 that the computed values of K_5^i and K_6^i are both lower than those of K_1^i and K_3^i, respectively. However, the differences are too small, in view of the large standard errors of the different binding constants, to be of significance.

Binding of MgADP to Av_1 and to the complex between Av_1 and Av_2

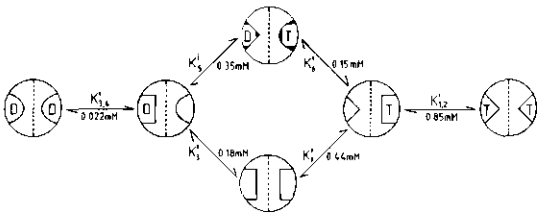
In a previous communication [16] we reported that we could not detect binding of MgATP to Av_1. In the same way as done for MgATP we tested MgADP for binding to Av_1. The flow dialysis results of Table 3 show that Av_1 does not bind MgADP. When the binding of MgADP to a 1:3 mixture of Av_1 and Av_2 was measured, it appeared that the presence of Av_1 has no effect on the binding of MgADP to Av_2 (Table 3). In these experiments the binding of MgADP to Av_2 was compared to the binding of MgADP to a mixture of Av_1 and Av_2 at three different nucleotide concentrations. This was done both for reduced and oxidized Av_2 and in all cases there was no appreciable difference in the binding of MgADP to Av_2 when Av_1 was present.

Table 3. *Binding of MgADP to Av_1 and to the complex of Av_1 and Av_2*
Data were obtained from flow dialysis experiments as described in Materials and Methods. The concentration of the proteins used in binding measurements to Av_1 or Av_2 alone were: 84 μM Av_1 and 63 μM Av_1 in the presence or absence, respectively, of 2 mM $Na_2S_2O_4$, 138 μM reduced Av_2 and 185 μM oxidized Av_2. Protein concentrations of the mixtures of Av_1 and Av_2 were 138 μM reduced Av_2 + 42 μM Av_1 and 185 μM oxidized Av_2 + 63 μM Av_1. Oxidation of Av_2 was performed as described in Materials and Methods. All proteins contained 50 mM Tes.NaOH, 5 mM $MgCl_2$ (pH 7.4) and, when indicated, also 2 mM $Na_2S_2O_4$. r = mol MgADP bound/mol Av_2

Protein	2 mM $Na_2S_2O_4$ present		$Na_2S_2O_4$ absent	
	free concentration of MgADP	r	free concentration of MgADP	r
	mM	mol/mol	mM	mol/mol
Av_1	0.2	0	0.2	0
Av_2	0.07	0.96	0.04	1.30
$Av_2 + Av_1$	0.07	0.96	0.04	1.28
Av_2	0.27	1.79	0.20	1.87
$Av_2 + Av_1$	0.28	1.68	0.20	1.87
Av_2	0.76	2.08	0.73	2.11
$Av_2 + Av_1$	0.76	2.09	0.76	1.97

General conclusion

Table 1 shows that the specific activity of Av_2 determines to a great extent the binding properties of the protein. However, it was discovered that there is no simple way to correct



Scheme 3. *Schematic representation of a conformational model of the binding of MgATP (T) and MgADP (D) to reduced Av_2*

the binding parameters obtained with a particular Av_2 preparation for inactive protein present. Thus we used Fe protein with the highest obtainable specific activity for binding measurements, although the data show differences between batches of enzyme. We have no explanation for this phenomenon yet.

Figs 1 and 2 show the saturation curves obtained for the binding of MgATP and MgADP, respectively, to the different oxidation states of Av_2. For oxidized Av_2 it was found that binding of the first adenine nucleotide has hardly if any effect on the affinity of the protein for a second adenine nucleotide. This holds for the binding of both MgATP and MgADP. For reduced Av_2, on the other hand, it was found that there is interaction between the binding sites. Binding of MgATP to reduced Av_2 induces negative co-operativity in the binding of a second molecule of MgATP, while binding of MgADP to reduced Av_2 shows a positive co-operative behaviour in the binding of a second molecule of MgADP. Since Dalziel and Engel [30] showed that a dimeric protein following the model of Monod, Wyman and Changeux [31] cannot exhibit negative co-operativity, it can be concluded that the model of Koshland, Némethy and Filmer [32] might be valid here.

The binding data obtained from the competition experiments were fitted to a two-site model as represented by Scheme 2, with identical binding sites for MgATP and MgADP present on the protein. To fit the model given in Scheme 2, the dissociation constants for the binding of MgATP and MgADP to the Fe protein were those determined from the binding experiments with MgATP and MgADP alone (Figs 1 and 2). It was found that the competition data can be explained in terms of a two-site model, using values for $K_5 = K_5^i$ and $K_6 = K_6^i$ (see Scheme 2) as given in Table 2. More complicated models are possible but mean that four, five or six parameters must be varied in computer analysis, compared to one parameter in the case of the simpler two-site model. Although these more complicated models may be fitted to the binding data, our experimental evidence, showing that in no case are more than two molecules of MgATP plus MgADP bound per molecule of Av_2, makes such models less attractive.

By using the hypothesis that initially the two adenine nucleotide binding sites on the Fe protein are identical, it is possible to convert the stoichiometric binding constants, as obtained from computer analysis of the different saturation curves, into site-directed or intrinsic binding constants. Table 2 summarizes the intrinsic dissociation constants as calculated for the case of two, initially identical, adenine nucleotide binding sites. In the case of oxidized Av_2 we suggest no significant interaction between the two binding sites. For reduced Av_2 the situation is different, because experimentally we found interaction between the two sites on binding MgATP or MgADP. The molecular events in the binding process of adenine nucleotides to reduced Av_2 are visualized in Scheme 3. Binding of MgATP to one site of the protein decreases the affinity of the second site for MgATP, but has no appreciable effect on the affinity for MgADP. Binding of one molecule of MgADP to the Fe protein stimulates the binding of a second molecule of MgADP and, to a much lesser extent, the binding of MgATP. The postulated model thus predicts that the conformation change on binding of MgATP to reduced Av_2 differs from that on binding of MgADP.

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REFERENCES

1. Mortenson, L. E. & Thorneley, R. N. F. (1979) *Annu. Rev. Biochem. 48*, 387–418.
2. Bui, P. T. & Mortenson, L. E. (1969) *Biochemistry 8*, 2462–2465.
3. Biggens, D. R. & Kelly, M. (1970) *Biochim. Biophys. Acta 205*, 288–299.
4. Tso, M.-Y. W. & Burris, R. H. (1973) *Biochim. Biophys. Acta 309*, 263–270.
5. Hageman, R. V., Orme-Johnson, W. H. & Burris, R. H. (1980) *Biochemistry 19*, 2333–2342.
6. Miller, R. W., Robson, R. L., Yates, M. G. & Eady, R. R. (1980) *Can. J. Biochem. 58*, 542–548.
7. Zumft, W. G., Palmer, G. & Mortenson, L. E. (1973) *Biochim. Biophys. Acta 292*, 413–421.
8. McKenna, C. E., Stephens, P. J., Eran, H., Luo, G. M., Matai Ding, F. X. Z. & Nguyen, H. T. (1984) in *Advances in nitrogen fixation research* (Veeger, C. & Newton, W. E., eds) pp. 115–122, Martinus Nijhoff, The Hague.
9. You, C. B., Wang, H. X., Ping, S. Z. & Gao, M. S. (1983) *Acta Phytophysiol. Sin 9*, 403–411.
10. Van Rossen, A. R., Peeters, J. M. F. & Heremans, K. A. H. (1973) *Arch. Int. Physiol. Biochem. 81*, 987.
11. Eady, R. R. & Smith, B. E. (1978) in *Dinitrogen fixation* (Hardy, R. W. F., ed.) pp. 399–490, Wiley-Interscience, New York.
12. Stephens, P. J., McKenna, C. E., McKenna, M. C., Nguyen, H. T. & Lowe, D. J. (1982) in *Electron transport and oxygen utilization* (Ho, C., ed.) pp. 405–409, MacMillan Press, New York.
13. You, C. B., Wang, H. X., Gao, M. S. & Ping, S. Z. (1984) in *Advances in nitrogen fixation research* (Veeger, C. & Newton, W. E., eds) p. 168, Martinus Nijhoff, The Hague.
14. Weston, M. F., Kotake, S. & Davis, L. C. (1983) *Arch. Biochem. Biophys. 225*, 809–817.
15. Ljones, T. & Burris, R. H. (1978) *Biochemistry 17*, 1866–1872.
16. Cordewener, J., Haaker, H. & Veeger, C. (1983) *Eur. J. Biochem. 132*, 47–54.
17. Thorneley, R. N. F. & Cornish-Bowden, A. (1977) *Biochem. J. 165*, 255–262.
18. Braaksma, A., Haaker, H., Grande, H. J. & Veeger, C. (1982) *Eur. J. Biochem. 121*, 483–491.
19. Goa, J. (1953) *Scand. J. Clin. Lab. Invest. 5*, 218–222.
20. Sedmak, J. J. & Grossberg, S. E. (1977) *Anal. Biochem. 79*, 544–552.
21. Braaksma, A., Haaker, H. & Veeger, C. (1983) *Eur. J. Biochem. 133*, 71–76.
22. Colowick, S. P. & Womack, E. C. (1969) *J. Biol. Chem. 244*, 774–777.
23. Hummel, J. P. & Dreijer, W. J. (1962) *Biochim. Biophys. Acta 63*, 530–532.
24. Williamson, J. R. & Corkey, B. E. (1969) *Methods Enzymol. 13*, 481–497.

25. Scatchard, G. (1949) *Ann. N. Y. Acad. Sci. 51*, 660–672.
26. Adams, E. Q. (1916) *J. Am. Chem. Soc. 38*, 1503–1510.
27. Mortenson, L. E., Walker, M. N. & Walker, G. A. (1976) in *Nitrogen fixation* (Newton, W. E. & Nijman, C. J., eds) pp. 117–149, Washington State University Press, Seattle.
28. Thorneley, R. N. F. & Eady, R. R. (1973) *Biochem. J. 133*, 405–408.
29. Cordewener, J., Haaker, H. & Veeger, C. (1984) in *Advances in nitrogen fixation research* (Veeger, C. & Newton, W. E., eds) p. 147, Martinus Nijhoff, The Hague.
30. Dalziel, K. & Engel, P. C. (1968) *FEBS Lett. 1*, 349–352.
31. Monod, J., Wyman, J. & Changeux, J.-P. (1965) *J. Mol. Biol. 12*, 88–118.
32. Koshland, D. E., Némethy, G. & Filmer, D. (1966) *Biochemistry 5*, 365–385.

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Chapter 4
Binding of ADP and orthophosphate during the ATPase reaction of nitrogenase

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The pre-steady-state ATPase activity of nitrogenase from *Azotobacter vinelandii* was investigated. By using a rapid-quench technique, it has been demonstrated that with the oxidized nitrogenase complex the same burst reaction of MgATP hydrolysis occurs as observed with the reduced complex, namely 6–8 mol orthophosphate released/mol MoFe protein. It is concluded that the pre-steady-state ATPase activity is independent of electron transfer from Fe protein to MoFe protein. Results obtained from gel centrifugation experiments showed that during the steady state of reductant-independent ATP hydrolysis there is a slow dissociation of one molecule of MgADP from the nitrogenase proteins ($k_{off} \leqslant 0.2\ s^{-1}$); the second MgADP molecule dissociates much faster ($k_{off} \geqslant 0.6\ s^{-1}$). Under the same conditions orthophosphate was found to be associated with the nitrogenase proteins. The rate of dissociation of orthophosphate from the nitrogenase complex, as estimated from the gel centrifugation experiments, is in the same order of magnitude as the steady-state turnover rate of the reductant-independent ATPase activity (0.6 mol P_i formed · s^{-1} · mol Av_2^{-1} at 22°C). These data are consistent with dissociation of orthophosphate or MgADP being rate-limiting during nitrogenase-catalyzed reductant-independent ATP hydrolysis.

The nitrogenase enzyme consists of two redox proteins, commonly named MoFe protein and Fe protein. Both proteins, plus MgATP and a source of electrons, are required for the reduction of N_2 and H^+ to NH_3 and H_2. Besides N_2, a wide range of alternative substrates are reduced by nitrogenase [1]. All these substrates have in common a triple bond. In the absence of any particular substrate, nitrogenase catalyzes the reduction of protons to H_2. Electron transfer to substrates is dependent upon MgATP hydrolysis. During electron transfer from reductant to substrates at least two molecules of MgATP are hydrolyzed to MgADP and orthophosphate per electron transferred [2].

Studies have been reported on the role of MgATP in the nitrogenase reaction [3–5]. Eady et al. [4] have shown that MgATP hydrolysis is coupled to the pre-steady-state electron transfer between the Fe protein and MoFe protein of *Klebsiella pneumoniae*. Hageman et al. [5] analyzed the stoichiometry of the rapid burst of MgATP hydrolysis in the pre-steady-state electron transfer reaction between the nitrogenase proteins of *Azotobacter vinelandii*. They found that about 2.4 molecules of MgATP are hydrolyzed per Fe protein molecule in the initial burst reaction. Assuming that in the pre-steady-state reaction one electron is transferred from the Fe protein to the MoFe protein, this indicates that the MgATP hydrolysis occurring in the steady-state reaction (2 ATP/e) can be all accounted for as occurring in a reaction coupled directly to electron transfer between the two nitrogenase proteins. However in the absence of a source of reducing equivalents, nitrogenases from different organisms have been shown to catalyze the hydrolysis of MgATP [2, 6–10]. This activity of nitrogenase is called the reductant-independent ATPase activity.

In this paper data are presented on the pre-steady-state ATPase activity of nitrogenase from *A. vinelandii*, both in the presence and absence of the electron donor dithionite. Furthermore experiments will be described that indicate that during reductant-independent steady-state MgATP hydrolysis ADP and phosphate bind to nitrogenase. The implications of these data for the mechanism of action of nitrogenase will be discussed.

MATERIALS AND METHODS

Preparation of nitrogenase proteins

Nitrogenase components were isolated as described earlier [11] from *A. vinelandii* ATCC 478 grown in a batch culture at low free oxygen concentration and harvested during the logarithmic growth phase. The specific activities of the purified preparations of Av_1 and Av_2 were, except when stated otherwise in the text or in the legends, at least 2000 nmol ethylene produced · min^{-1} · (mg protein)$^{-1}$. Fe protein was isolated from regularly grown cells and not from O_2-shocked cells [12]. The Fe/S content of Fe protein varied between 3–4 mol/mol.

The molar concentrations of Av_1 and Av_2 were calculated from the molecular masses of 220 kDa and 63 kDa, respectively. Oxidation of Av_2 was performed with phenazine

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Abbreviations. The MoFe and Fe proteins of the nitrogenases of *Azotobacter vinelandii*, *Clostridium pasteurianum* and *Klebsiella pneumoniae* are referred to as Av_1 and Av_2, Cp_1 and Cp_2 and Kp_1 and Kp_2, respectively.

methosulphate as oxidant [13]. Av_1 was oxidized with solid thionine as oxidant [14] and separated from the excess thionine on a Bio-Gel P-6DG column.

Enzymatic assays

Unless otherwise specified, the nitrogenase activity (acetylene reduction) was measured at pH 7.4 and 30°C under the standard assay conditions as described by Braaksma et al. [15].

The reductant-dependent steady-state ATPase activity was measured under the same experimental conditions as used for the acetylene reduction assay. Samples (10 µl) were removed at 2-min intervals from the reaction mixture by a gas-tight syringe and transferred to a tube containing 0.2 M NaOH (90 µl). The samples were then assayed for creatine by the method of Ennor [16].

Reductant-independent steady-state ATPase activity was assayed in glass cuvettes (1-cm light path) capped with a Subaseal rubber closure to allow anaerobic spectrophotometry. The reaction mixture (1 ml) contained: 50 mM Tes/NaOH, 40 mM α-D-glucose, 2 mM ATP, 5 mM $MgCl_2$, 2.5 mM phospho*enol*pyruvate, 0.1 mM NADH, 0.01 mg/ml lactate dehydrogenase (≈ 550 U/mg), 0.01 mg/ml pyruvate kinase (≈ 200 U/mg), final pH 7.4. The cuvettes with reaction mixture were made anaerobic by evacuating and refilling with argon five times. Trace amounts of O_2 were removed by adding glucose oxidase (final concentration 1 mg/ml) to the reaction mixture. After 5 min, one of the (oxidized) nitrogenase components was injected with a gas-tight syringe and, after preincubation (22°C for 3 min), the reaction was started by adding the complementary (oxidized) protein. The oxidation of NADH was followed by monitoring the absorbance at 340 nm.

Analytical methods

Protein concentration was estimated by the microbiuret method of Goa [17] calibrated with bovine serum albumin. The concentration of bovine serum albumin was estimated by its absorbance at 279 nm ($\varepsilon_{279\,nm} = 45.36 \times 10^6\ M^{-1} \cdot cm^{-1}$). Phosphate was measured colorimetrically as described by Ottolenghi [18] using a modification of the method of Baginski et al. [19]. ATP and ADP concentrations were determined enzymatically as described by Williamson and Corkey [20].

Rapid-quench technique

The rapid-quenching apparatus (Update Instruments Inc.) used to study the pre-steady-state burst of MgATP hydrolysis by nitrogenase was of the same design as described by Lowe and Thorneley [21]. The method involves rapid mixing of two solutions to start the reaction. The reaction time, which is determined by the time interval between mixing and quenching, can be controlled by changing the flow rate (the Ram speed of the apparatus) and/or the length of the tube connecting the mixing chamber and the end of the delay pipe. In this way, acid-quenched reaction mixtures were obtained with reaction times varying between 17 ms and 650 ms.

One syringe of the rapid-quenching apparatus contained the nitrogenase proteins at various concentrations (20–80 µM Av_1, 50–200 µM Av_2) in 50 mM Tes/NaOH, 5 mM $MgCl_2$, 5 mM $Na_2S_2O_4$, final pH 7.4. The other syringe contained 10 mM MgATP in 50 mM Tes/NaOH, 5 mM $Na_2S_2O_4$, final pH 7.4. In experiments with dye-oxidized

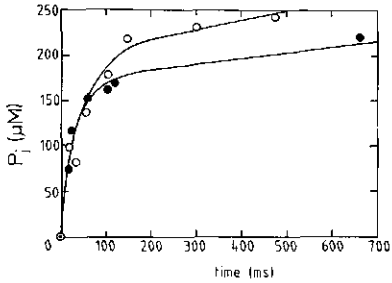

Fig. 1. *Measurement of the pre-steady-state burst of P_i formation from MgATP by nitrogenase.* The time course of P_i release on mixing equal volumes of nitrogenase protein and MgATP was measured during the first second of the reaction. The experimental conditions used for the rapid-quench measurements are described in Materials and Methods. Nitrogenase concentrations after mixing were, in the experiment with reduced proteins (○——○), 25 µM Av_1 and 49.7 µM Av_2, and in the experiment with dye-oxidized proteins (●——●), 26.9 µM Av_1 and 46 µM Av_2

nitrogenase proteins, $Na_2S_2O_4$ was omitted from the solutions. All rapid-quench experiments were performed at 22°C. The concentrations of the nitrogenase proteins used are given in the legends to the figures and the tables. Each datum point on a reaction progress curve was obtained by injecting a given volume of the reaction mixture (approximately 0.4 ml) into 0.4 ml 10% (w/v) trichloroacetic acid, contained in a stoppered test tube and stored on ice until analyzed for P_i. The exact volume of the reaction mixture was determined by weighing. The denatured protein was precipitated by centrifugation, and P_i was measured in the supernatant by the method of Ottolenghi [18]. P_i released was corrected for the background P_i in the assay mixture, caused by non-enzymatic hydrolysis of MgATP. Data points were measured in triplicate.

Detection of protein-bound ligands using gel centrifugation

Oxidized nitrogenase proteins (2.5–80 µM Av_1, 25–200 µM Av_2 in 50 mM Tes/NaOH, 5 mM $MgCl_2$, final pH 7.4) were incubated as indicated with MgATP, MgADP or KP_i at 22°C. Protein-bound ligands were detected with gel centrifugation as described by Penefsky [22]. 0.1 ml of the reaction mixture was applied to a pre-cooled (4°C) molecular sieve column, consisting of 2 ml (after centrifugation) of Sephadex G-50 (medium or fine as indicated) packed in a 2-ml plastic syringe and equilibrated with 50 mM Tes/NaOH, 100 mM NaCl, final pH 7.4. The column was placed in a test tube containing 0.1 ml 10% (w/v) trichloroacetic acid for the determination of P_i and 0.1 ml 10% (v/v) perchloric acid for the determination of adenine nucleotides. Thus the effluent of the column was quenched directly during centrifugation (1 min, 900 × *g* at the tip of the syringe). The volume of the effluent was determined and aliquots were taken for the measurement of P_i and/or adenine nucleotides. The amount of precipitated protein was also determined.

Materials

All chemicals used were of the purest grade available and were obtained from commercial sources. The enzymes lactate dehydrogenase, pyruvate kinase and glucose oxidase were purchased from Boehringer.

Table 1. *Rapid-quench measurements of the P_i production during the initial burst of MgATP hydrolysis by nitrogenase*
The rapid-quench experiments were performed with dithionite-reduced nitrogenase proteins, or with oxidized proteins (*) as described in Materials and Methods. The specific activities of Av_1 and Av_2 (nmol ethylene produced · min^{-1} · mg $protein^{-1}$) were measured under standard conditions as described in Materials and Methods. The amount of P_i produced in the burst reaction was determined as described in Materials and Methods

Expt	Specific activity		Ratio of the proteins Av_2/Av_1	Concn in the reaction mixture		P_i produced in the pre-steady-state burst reaction	P_i produced/Av_1
	Av_1	Av_2		Av_1	Av_2		
	nmol · min^{-1} · mg^{-1}			μM			mol/mol
1.	1600	1200	1.9	22.8	43.9	71.6	3.1
2.	1600	1200	1.9	22.9	43.9	77.6	3.4
3.	1600	1200	1.9	22.9	43.9	67.8	3.0
4.	1800	1700	2.4	15.8	37.3	37.3	2.4
5.	1800	1400	2.9	7.7*	22.4*	25.3	3.3
6.	1700	1700	3.0	15.2	45.9	70.6	4.6
7.	1800	1200	9.9	10.1	100.0	68.0	6.7
8.	1400	2000	1.7	26.9*	46.0*	172.2	6.4
9.	2000	2000	2.0	25.0	49.7	137.5	5.5
10.	2000	2000	2.0	25.0	49.7	196.6	7.9
11.	2500	2000	6.9	24.0	165.0	113.2	4.7

RESULTS

ATP is hydrolyzed during nitrogenase catalysis. Eady et al. [4] showed that MgATP hydrolysis and electron transfer from Fe protein to MoFe protein have the same time constants and Thorneley [23] showed that there is no electron transfer in absence of MgATP. These observations strongly suggest that MgATP hydrolysis is coupled to electron transfer. In Fig. 1, it is shown that when the proteins are dye-oxidized, and therefore no electrons are available to be transferred between the two proteins, the burst of MgATP hydrolysis also occurs. This shows that pre-steady-state MgATP hydrolysis is independent of electron transfer. These experiments were performed at 22 °C and at this temperature the rate of MgATP hydrolysis was too fast compared to the dead-time of the rapid quench apparatus used to allow the determination of the rate constant for MgATP hydrolysis. Although the rate constant cannot be determined, the data show that there is no significant difference in the rates of the pre-steady-state MgATP hydrolysis by either the oxidized or the reduced proteins. These observations strongly suggest that MgATP hydrolysis is not coupled to electron transfer, but precedes a fast electron transfer between the components.

The stoichiometry of the pre-steady-state MgATP hydrolysis in relation to the specific activity of Fe protein and to the ratio of Fe protein to MoFe protein was determined. These results are presented in Table 1. The experiments are arranged into two groups. The group of experiments with Fe protein having a specific activity of ≤ 1700 nmol · min^{-1} · mg^{-1} and with a ratio of Fe protein/MoFe protein between 1.9 and 3.0 gave a stoichiometry of 3.3 ± 0.7 mol P_i formed/mol Av_1 (experiments 1–6).

Experiments with Fe protein with a specific acivity of 2000 nmol · min^{-1} · mg^{-1} gave a value of 6.6 ± 1.2 mol/mol (experiments 8–10). In both cases the values are independent of the oxidation state of the proteins. Also with protein with a lower specific activity, the P_i production in the burst reaction can be the same as with protein with a high specific activity. This occurs at a high ratio of Fe protein to MoFe protein (experiment 7). When the specific activity of Fe protein is high, ratios of Fe protein/MoFe protein above 2 do not increase the P_i production in the burst reaction (experiment 11). When the ratio of Fe protein/MoFe protein decreases below 2, the P_i production in the burst reaction declines (not shown).

From the experiments described it can be concluded that Fe protein with a specific activity of 2000 nmol · min^{-1} · mg^{-1} at a ratio of 2 mol/mol with respect to MoFe protein gives the maximal P_i production in the burst reaction. Higher ratios do not increase the P_i production. With less active protein it is possible to increase the P_i production by increasing the ratio of the proteins.

The effect of flavodoxin on the burst reaction was also studied. In experiments 1, 3, 6 and 9 respectively 100 μM, 100 μM, 98 μM and 670 μM flavodoxin was present. Flavodoxin did not affect the P_i production in the pre-steady-state ATPase reaction, neither did the redox state of the protein (experiments 5 and 8).

The pre-steady-state ATPase reaction is followed by a slower steady-state ATPase reaction. This type of kinetic behaviour is indicative for the formation of an intermediate, especially in the case of oxidized proteins where no other events, e.g. electron transfer, can take place. In the currently accepted mechanism for electron transfer through nitrogenase, the intermediate built up during catalysis is thought to be the complex of oxidized Fe protein with (partly) reduced MoFe protein [24]. Since it is shown here that the burst reaction is independent of electron transfer (oxidized proteins give the same pre-steady-state ATPase activity as reduced proteins; see Fig. 1 and Table 1), the intermediate built up in the case of oxidized proteins cannot be the complex between oxidized Fe protein and reduced MoFe protein. A possible candidate for the intermediate under these conditions might be a MgADP-protein complex in which case the rate of dissociation of MgADP is the slowest step in the catalytic cycle, or a phosphate-protein complex in which the rate of dissociation of phosphate is rate-limiting. To test these two hypotheses the formation of enzyme intermediates was studied.

The method used was gel centrifugation. With this method one can relatively rapidly separate proteins from incubation mixtures. By using this technique, covalently or tightly bound ligands have been detected [22]. Nitrogenase proteins were

Table 2. *Binding of adenine nucleotides to nitrogenase during the steady-state ATPase activity*
Binding of MgATP and MgADP to the nitrogenase proteins was determined under different incubation conditions using the gel centrifugation technique described in Materials and Methods, with Sephadex G-50 fine as molecular sieve. Av_1 and Av_2 were oxidized as described in Materials and Methods. Experiments with the nitrogenase proteins under reducing conditions (*) were performed with 2 mM $Na_2S_2O_4$ in the incubation mixture. In that case the gel centrifugation columns also were run anaerobically with 2 mM $Na_2S_2O_4$ present in the equilibration buffer. The concentration of adenine nucleotides was determined enzymatically [20] as described in Materials and Methods

Expt	Starting concentrations				Reaction time	Concn at the time of separation		Adenine nucleotides associated with the proteins	
	Av_1	Av_2	ATP	ADP		ATP	ADP	ATP	ADP
	μM		mM		min	mM		mol/mol Av_2	
1.	25	50	8.4	0.2	0	7.9	0.4	0	1.1
2.	25	50	8.4	0.2	1	6.6	1.6	0	0.9
3.	25	50	8.4	0.2	4	4.5	4.2	0	1.1
4.	9.2	80	10.0	0.3	1	8.0	2.0	0	1.3
5.	0	50	8.4	0.2	1	8.4	0.2	0.9	0.4
6.	25	50	0.2	9.0	4	0.2	8.8	0	1.0
7.	0	100	0.2	7.1	1	0.2	6.8	0	0.9
8.*	25	50	8.4	0.2	1	4.2	3.9	0	0.4
9.*	25	50	0.2	10.5	1	0.2	10.3	0	0.7
10.*	0	100	8.4	0.2	1	8.4	0.2	0.6	0.1
11.*	0	100	0.2	8.0	1	0.2	7.4	0	0.7

Table 3. *Acid-labile P_i associated with the nitrogenase proteins after incubation with MgATP and P_i under different conditions*
Binding of P_i to the nitrogenase proteins was determined using the gel centrifugation technique as described in Materials and Methods, with Sephadex G-50 medium as molecular sieve. Av_1 and Av_2 were oxidized as described in Materials and Methods. The amount of acid-labile phosphate in the eluent was determined by the method of Ottolenghi [18]

Expt	Starting concentrations					Reaction time	Concn at the time of separation			P_i concn in the protein fraction after gel centrifugation
	Av_1	Av_2	ATP	ADP	P_i		ATP	ADP	P_i	
	μM		mM			min	mM			μM
1.	72	–	–	–	9	3	–	–	9	20
2.	–	185	–	–	9	3	–	–	9	23
3.	72	185	–	–	9	3	–	–	9	29
4.	63	185	16.7	0.4	0.3	1	15.1	2.0	2.1	59
5.	63	185	16.7	0.4	0.3	3	12.7	4.1	4.9	87
6.	–	–	–	–	4.5	–	–	–	4.5	14
7.	–	–	–	–	9	–	–	–	9	25

incubated at 22 °C and the reactions were slowed down during the separation by loading the protein mixture on a pre-cooled column. Within approximately 5 s with Sephadex G-50 medium and 15 s with Sephadex G-50 fine, the protein separated from the incubation mixture and was quenched in acid. The protein fractions were analyzed for P_i and adenine nucleotides. The results are presented in the Tables 2 and 3. When nitrogenase was catalyzing reductant-independent ATP hydrolysis, gel centrifugation experiments indicated that ADP was bound to the protein irrespective of the ATP/ADP ratio of the incubation mixture (Table 2, experiments 1–4). Since during a gel centrifugation experiment with Sephadex G-50 fine the proteins are separated from the incubation mixture in about 2 s, and it takes approximately 15 s to travel through the rest of the column, only ligands with a rate constant of dissociation below 0.2 s^{-1} will be detected in the protein fraction. This means that, in the absence of the components of the reaction mixture, dissociation of tightly bound MgADP from nitrogenase generated during turnover is a relatively slow step. Experiments 1 and 4 (Table 2) show in addition that there is no ATP present in the protein fractions after gel centrifugation of the incubation mixtures containing two different ratios of the nitrogenase proteins and a high concentration of MgATP. Only ADP was found to be associated with the proteins under all these conditions. This could mean that during gel centrifugation MgATP dissociates from the proteins, or that MgATP is hydrolyzed to MgADP and P_i. Only when oxidized Av_2 alone was incubated with MgATP, could binding of MgATP be detected (experiment 5). This indicates that the rate of dissociation of MgATP from oxidized Av_2 is slow enough to be detected by gel centrifugation. This makes it more likely that when Av_2 is incubated with MgATP in the presence of Av_1, the bound MgATP is very rapidly hydrolyzed to MgADP and P_i. The incubations of the nitrogenase proteins or Av_2 alone with MgADP (experiment 6 and 7 respectively) show that turnover conditions are not necessary for the observed tight binding of MgADP to Av_2. We also tested the effect of dithionite on the binding of MgATP and MgADP to the reduced nitrogenase proteins. Under all the conditions tested (experiments 8–11), less

adenine nucleotides were found to be associated with the proteins after gel centrifugation compared with similar incubations with the oxidized proteins: experiments 3 and 8 (turnover conditions); experiments 5 and 10 (MgATP binding to Av_2); experiments 6 and 9 (MgADP binding to Av_1 plus Av_2); experiments 7 and 11 (MgADP binding to Av_2). No MgATP or MgADP binding to Av_1 was detected (not shown).

We also tested binding of P_i to the nitrogenase proteins. No significant binding of P_i to nitrogenase could be detected in the experiments described in Table 2. However, when instead of Sephadex G-50 fine, Sephadex G-50 medium was used, which results in a faster elution of the proteins (approximately 5 s instead of 15 s), significant binding of P_i to nitrogenase could be detected (Table 3). A disadvantage of using Sephadex G-50 medium is that depending on the concentration of P_i in the incubation mixture, some P_i elutes from the column (see experiments 6 and 7).

Therefore gel centrifugation experiments with nitrogenase proteins under different incubation conditions must always be compared with control experiments using the same concentrations of P_i in the incubation mixture. This problem was not observed in the experiments with Sephadex G-50 fine. Table 3 shows that oxidized Av_1 or Av_2 when incubated, together or separately, with P_i, do not bind more P_i than the background (experiments 1–3 and 7 respectively). However, when oxidized Av_1 and Av_2 are incubated together with MgATP, the amount of P_i associated with the proteins is significantly above the background (compare experiments 4 and 5 with 6). These results indicate that under conditions were ATP is hydrolyzed (both nitrogenase components and MgATP present) P_i is bound to nitrogenase.

DISCUSSION

Two important observations are presented in this paper. (a) The pre-steady-state ATPase activity of nitrogenase is independent of electron transfer from Fe protein to MoFe protein. (b) MgADP and P_i remain bound to nitrogenase following MgATP hydrolysis. Both observations will be discussed in relation to the mechanism of action of nitrogenase.

It has been shown that the pre-steady-state burst of ATP hydrolysis has the same rate constant as electron transfer from Fe protein to MoFe protein [4] and electron transfer does not occur without MgATP [23]. Since it has now been shown that the pre-steady-state rate of MgATP hydrolysis is independent of the redox state of the proteins, this must mean that MgATP hydrolysis precedes a rapid initial electron transfer. Subsequently a slow rate-limiting process occurs.

In the currently accepted model, the rapid pre-steady-state rate of MgATP hydrolysis is tightly coupled to electron transfer from Fe protein to MoFe protein and the slower steady-state rate of MgATP hydrolysis is a consequence of the rate of re-reduction of oxidized Fe protein being relatively slow [5, 24]. This model does not predict a pre-steady-state burst of ATP hydrolysis for oxidized proteins. Since we have shown that the pre-steady-state ATPase activity of nitrogenase is independent of the redox state of the proteins, this means that the explanation for the observed slow steady-state ATPase activity cannot be due to the rate of reduction of oxidized Fe protein being rate-limiting in the ATPase activity.

Two possible explanations for the slow steady-state rate of MgATP hydrolysis by nitrogenase and in particular the reductant-independent ATPase activity, will be discussed. Firstly, during catalysis after the initial hydrolysis of MgATP the dissociation rate of MgADP from oxidized Fe protein or from the complex is slow and rate-limiting. With oxidized proteins this limits the reductant-independent ATPase activity, which for a 1:2 ratio of Av_1 to Av_2 is 0.6 mol P_i formed · s^{-1} · mol Av_2^{-1} at 22°C (J. Cordewener, unpublished results). Under the same conditions but in the presence of a saturating concentration of dithionite, the ATPase activity is 4.4 mol P_i formed · s^{-1} · mol Av_2^{-1}.

Unfortunately there are only indirect measurements of the rate of dissociation of MgADP from Fe protein. Thorneley and Cornish-Bowden [25] estimated from inhibition studies of MgADP on the MgATP-induced electron transfer from Kp_2 to Kp_1 that the rate constant of dissociation of MgADP from reduced Kp_2 was comparable with the rate of electron transfer, i.e. with $k = 2 \times 10^2\ s^{-1}$. There is no information about the rate of dissociation of MgADP from oxidized Fe protein or from oxidized Fe protein bound to MoFe protein. Our gel centrifugation experiments give information about the rate of dissociation of MgATP and MgADP from the nitrogenase proteins. Fe protein, free or bound to MoFe protein, was incubated at high MgATP and MgADP concentrations; conditions under which the two adenine nucleotide binding sites of the Fe protein were saturated [13]. Under all conditions gel centrifugation experiments showed that approximately 1 mol MgADP or MgATP was bound/mol Av_2 (see Table 2). In experiments with Sephadex G-50 fine, separation of proteins from the incubation mixture takes place in approximately 15 s. Thus only ligands with a rate constant of dissociation of less than 0.2 s^{-1} will be detected. This means that one adenine nucleotide molecule has a rate constant of dissociation of less than 0.2 s^{-1}; furthermore, the second MgADP bound to Av_2 must have a rate constant of dissociation of at least 0.6 s^{-1} (the rate constant of reductant-independent ATPase activity) because it cannot be detected by this method. It is therefore still possible that the rate of dissociation from Fe protein of the faster dissociating MgADP determines the steady-state rate of reductant-independent ATPase activity.

An alternative explanation for the observed low rate of steady-state ATPase activity compared to the rapid pre-steady-state rate is that in the pre-steady-state burst reaction, P_i becomes tightly or covalently bound to the proteins. The slow rate of dissociation or dephosphorylation determines the steady-state rate of MgATP hydrolysis. Our gel centrifugation experiments indicate that during turnover binding of P_i to nitrogenase can be detected (see Table 3). The fact that MgADP or MgATP binding was detectable both with Sephadex G-50 medium and fine, while P_i binding could only be demonstrated with Sephadex G-50 medium, must mean that the rate constant of dissociation of P_i from the nitrogenase complex is faster than that of MgADP or MgATP, but still in the range of 0.6 s^{-1}.

The stoichiometry of the pre-steady-state ATP hydrolysis (Table 1, experiments 8–10) might suggest the formation of a complex of 6–8 P_i molecules bound to a 1:2 complex of MoFe protein and Fe protein. The higher values were observed with Fe protein with higher activity. Since Fe protein has two MgATP binding sites [13], it means that when each binding site is converted into a catalytic site in the complex, each site has two turnovers in the burst reaction. Since one of the two adenine-nucleotide-binding sites has a low rate of dissociation of MgADP, it might be possible that this site will be constantly occupied by MgADP after the first molecule of MgATP is hydrolyzed in the pre-steady-state burst reaction. The observed hydrolysis of the other MgATP molecules then

takes place at the second low-affinity MgADP binding site. Although the rate of dissociation of P_i from this complex could determine the steady-state rate of reductant-independent ATP hydrolysis, it cannot be excluded that dissociation of MgADP from its low-affinity site is also involved. In the presence of an electron donor, dissociation of MgADP, P_i or dephosphorylation occurs at a higher rate.

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REFERENCES

1. Burns, R. C. & Hardy, R. W. F. (1975) in *Nitrogen fixation in bacteria and higher plants*, pp. 71–72, Springer, New York.
2. Ljones, T. & Burris, R. H. (1972) *Biochim. Biophys. Acta 275*, 93–101.
3. Ljones, T. (1979) *FEBS Lett. 98*, 1–8.
4. Eady, R. R., Lowe, D. J. & Thorneley, R. N. F. (1978) *FEBS Lett. 95*, 211–213.
5. Hageman, R. V., Orme-Johnson, W. H. & Burris, R. H. (1980) *Biochemistry 19*, 2333–2342.
6. Bui, P. T. & Mortenson, L. E. (1969) *Biochemistry 8*, 2462–2465.
7. Hadfield, K. L. & Bulen, W. A. (1969) *Biochemistry 8*, 5103–5108.
8. Jeng, D. Y., Morris, J. A. & Mortenson, L. E. (1970) *J. Biol. Chem. 245*, 2809–2813.
9. Mortenson, L. E., Walker, M. N. & Walker, G. A. (1976) in *Proceedings of the 1st international symposium on nitrogen fixation* (Newton, W. E. & Nijman, C. J., eds) vol. 1, pp. 117–149, Washington State University Press, Pullman WA.
10. Iman, S. & Eady, R. R. (1980) *FEBS Lett. 110*, 35–38.
11. Cordewener, J., Haaker, H. & Veeger, C. (1983) *Eur. J. Biochem. 132*, 47–54.
12. Haaker, H., Cordewener, J., Ten Asbrock, A., Wassink, H., Eady, R. & Veeger, C. (1985) in *Nitrogen fixation research progress* (Evans, H. J., Bottomley, P. J. & Newton, W. E., eds) pp. 567–576, Martinus Nijhoff Publishers, Dordrecht.
13. Cordewener, J., Haaker, H., Van Ewijk, P. & Veeger, C. (1985) *Eur. J. Biochem. 148*, 499–508.
14. Johnson, M. K., Thomson, A. J., Robinson, A. E. & Smith, B. E. (1981) *Biochim. Biophys. Acta 671*, 61–70.
15. Braaksma, A., Haaker, H., Grande, H. J. & Veeger, C. (1982) *Eur. J. Biochem. 12*, 483–491.
16. Ennor, A. H. (1957) *Methods Enzymol. 3*, 850–861.
17. Goa, J. (1953) *Scand. J. Clin. Lab. Invest. 5*, 218–222.
18. Ottolenghi, P. (1975) *Biochem. J. 151*, 61–66.
19. Bøginski, E. S., Foa, P. P. & Zak, B. (1967) *Clin. Chim. Acta 15*, 155–158.
20. Williamson, J. R. & Corkey, B. E. (1969) *Methods Enzymol. 13*, 481–497.
21. Lowe, D. J. &Thorneley, R. N. F. (1984) *Biochem. J. 224*, 877–886.
22. Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891–2899.
23. Thorneley, R. N. F. (1975) *Biochem. J. 145*, 391–396.
24. Thorneley, R. N. F. (1983) *Biochem. J. 215*, 393–403.
25. Thorneley, R. N. F. & Cornish-Bowden, A. (1977) *Biochem. J. 165*, 255–262.

Chapter 5
The role of MgATP-hydrolysis in nitrogenase catalysis

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5.1. Summary

The kinetic properties of MgATP hydrolysis by nitrogenase of Azotobacter vinelandii were investigated in the presence and in the absence of reducing equivalents. By measuring the ATPase activity of dye-oxidized nitrogenase proteins it is excluded that reductant-independent ATPase activity is a result of futile cycling of electrons. The turnover rates of Av_1 during reductant-dependent and reductant-independent ATPase activity, when measured with excess Av_2, have approximately the same value, i.e. 5 s^{-1} at pH 7.4 and 22°C, assuming the hydrolysis of 4 molecules of MgATP per turnover of Av_1. For Av_2, on the other hand, the maximum turnover rate during reductant-independent ATPase activity is only about 6% of that of reductant-dependent ATPase activity. While the reductant-dependent ATPase activity shows a sigmoidal dependence on the concentration of MgATP, the reductant-independent ATPase activity yields hyperbolic saturation curves. To account for these results two mechanisms will be discussed. One mechanism proposing that the rate-limiting step during nitrogenase catalyzed MgATP hydrolysis is the dissociation of MgADP from Av_2 and another mechanism assuming that the rate of dissociation of Av_1 from the complex Av_1 - Av_2 MgATP is larger with oxidized proteins compared with that of reduced proteins.

5.2. Introduction

Nitrogenase is a complex of two redox proteins: a tetrameric MoFe protein carrying the substrate-reducing site and a dimeric Fe protein. Both proteins are required for catalytic activity. Nitrogenase catalyses the reduction of N_2 to NH_3 in a reaction coupled with the hydrolysis of MgATP to MgADP and P_i. In addition to MgATP an anaerobic environment and a low-potential electron donor are obligatory for substrate reduction. In the absence of a source of reducing equivalents, nitrogenase still catalyses the hydrolysis of MgATP [1-6], the so-called reductant-independent ATPase activity. Orme-Johnson and Davis [7] gave an explanation for this activity. They suggested that after MgATP-dependent electron transfer from the Fe protein to the MoFe protein, the electron returns to the Fe protein, thus completing a futile cycle of MgATP hydrolysis but not resulting in substrate reduction. In contrast to studies on the mechanism of electron transfer inside the nitrogenase complex, those on the properties of the ATPase activity of nitrogenase are relatively rare, especially reports on the reductant-independent ATPase activity.

Some earlier studies on the reductant-independent ATPase activity were performed before pure nitrogenase proteins were available [2-4]. More recent reports on the properties of the reductant-independent ATPase activity concerns nitrogenase from Clostridium pasteurianum [5] and from Klebsiella pneumoniae [6]. The rate of reductant-independent ATP hydrolysis by nitrogenase was shown to be dependent on the ratio of the component proteins [5]. Both Cp and Kp nitrogenase showed a marked increase in ATPase activity when the pH was decreased below pH 7.0, with a maximum activity around pH 5.4 [refs. 2,6].

Little information is available about the relationship between the reductant-independent and the reductant-dependent ATPase activity. Is MgATP in both cases hydrolyzed at the same sites and by which way is MgATP hydrolysis coupled with electron transfer? How can electron transfer stimulate MgATP hydrolysis?

In this paper data will be presented that give an answer to some of those questions. A study was made of the dependence of both ATPase activities on the MgATP-concentration and on the component ratio comparing the relation between ATPase activity and substrate reduction activity. Two models will be presented which could explain the experimental data.

5.3. Materials and Methods

Preparation of nitrogenase proteins

Nitrogenase components were isolated as described earlier [8] from A.vinelandii ATCC 478 grown in a batch culture and harvested during the logarithmic growth phase. The specific activity of the purified preparations of Av_1 and Av_2 were, except when stated otherwise in the text or in the legends, at least 2000 nmol of ethylene produced.min^{-1}.(mg protein)$^{-1}$ The molar concentrations of Av_1 and Av_2 were calculated by using a relative molecular mass of 220 kDa and 63 kDa, respectively. Oxidation of Av_2 was performed with phenazine methosulphate as oxidant [9]. Av_1 was oxidized with solid thionine as oxidant [10] and separated from the excess thionine on a Bio-Gel P-6DG column. Partial inactivation of one the nitrogenase proteins was performed by incubating the protein with various amounts of O_2-saturated buffer.

Enzymatic assays

Unless otherwise specified, the nitrogenase activity (acetylene reduction) was measured at pH 7.4 and 30°C under the standard assay conditions as described by Braaksma et al. [11].

The reductant-dependent ATPase activity was measured under the same experimental conditions as used for the acetylene reduction assay. Samples (10 µl) were removed at 2-minute intervals from the reaction mixture by a hypodermic syringe and transferred to a tube containing 0.2 N NaOH (90 µl). The samples were then assayed for creatine by the method of Ennor [12].

Reductant-independent ATPase activity was assayed at 22°C in glass cuvettes (1-cm light path) capped with a Subaseal rubber closure to allow anaerobic spectrophotometry. The reaction mixture (1 ml) contained: 50 mM Tes/NaOH, 40 mM α-D-glucose, 2 mM ATP, 5 mM $MgCl_2$, 2.5 mM phosphoenolpyruvate, 0.1 mM NADH, 0.01 mg/ml lactate dehydrogenase (ca. 550 U/mg), 0.01 mg/ml pyruvate kinase (ca. 200 U/mg), final pH 7.4. The cuvettes with reaction mixture were made anaerobically by evacuating and refilling with argon five times. Trace amounts of O_2 were removed by adding glucose oxidase (final concentration 1 mg/ml) to the reaction mixture. After 5 min, one of the (oxidized) nitrogenase components was injected with a gas-tight syringe and, after preincubation (22°C for 3 min), the reaction was started by

adding the complementary (oxidized) protein. The oxidation of NADH was followed by monitoring the absorbance at 340 nm.

Analytical methods

Protein concentration was estimated, after denaturation and precipitation of the protein with DOC and TCA [13], by the microbiuret method [14] using bovine serum albumin as standard. Phosphate was measured colorimetrically as described by Ottolenghi [15] using a modification of the method of Baginski et al. [16].

Materials

All chemicals used were of the purest grade available and were obtained from commercial sources. The enzymes lactate dehydrogenase, pyruvate kinase and glucose oxidase were purchased from Boehringer.

5.4. Results

A study of the involvement of MgATP in nitrogenase catalysis is hindered by the fact that no MgATP hydrolysis is catalysed by the separate components. Binding experiments to the individual nitrogenase proteins showed that Fe protein, but not MoFe protein binds MgATP [8]. However, MgATP hydrolysis occurs only when MoFe protein interacts with the Fe protein-MgATP complex. Under experimental conditions optimal for substrate reduction, the ATPase activity is coupled to the rate of substrate reduction by the nitrogenase complex, with a minimal hydrolysis of two molecules of MgATP per electron transferred to substrate [1,17-20]. Under suboptimal conditions much higher values than 4 were reported for the ATP:$2e^-$ ratio [6,21].

In addition to results published on the ATPase activity of nitrogenase depending upon component ratio [1], temperature [19], inhibition by antibody [22], heterogeneous components [21], we report here the relationship between the specific activities of the component proteins and the ATPase activity of the complex (Fig. 1). In case of Av_2 the linear relationship between nitrogenase activity and ATPase activity suggests a direct coupling between electron transfer from Fe protein to MoFe protein and the ATPase activity of nitrogenase. When Av_1 is partially inactivated by O_2, the

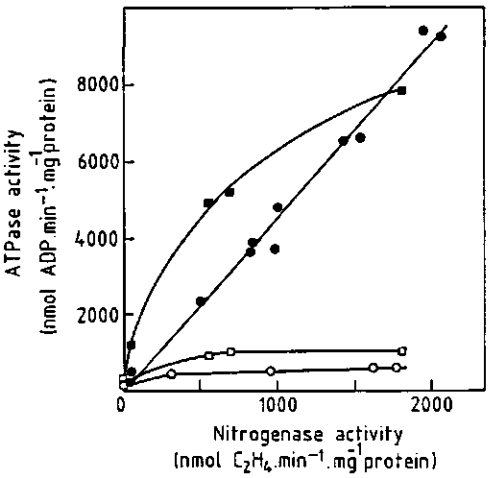

Fig. 1. ***Relationship between nitrogenase activity and reductant-dependent and reductant-independent ATPase activity.***

Either Av_1 or Av_2 was partly inactivated by exposing to O_2 for a limited period and the standard assay carried out in the presence of the other component not being exposed to O_2.

Assays for nitrogenase activity and reductant-dependent ATPase activity were performed as described in Materials & Methods.

Production of ethylene and creatine was measured on the same reaction vial. Reaction conditions were 25 mM Hepes/KOH, 10 mM $MgCl_2$, 5 mM ATP, 10 mM creatine-P, 1 mg/ml bovine serum albumin, 0.2 mg/ml creatine kinase, 20 mM $Na_2S_2O_4$, final pH 7.4. Measurement of the reductant-independent ATPase activity was performed as described in Materials & Methods. Nitrogenase activity and ATPase activity are expressed per mg Av_1 (■-■, □-□) and per mg Av_2 (●-●, o-o).

Closed symbols are reduced proteins, open symbols are oxidized proteins. (●-●), 1.1 μM Av_1 and 1.1 μM Av_2; (■-■), 0.46 μM Av_1 and 2.2 μM Av_2; (o-o), 1 μM Av_1 and 1.1 μM Av_2; (□-□), 0.23 μM Av_1 and 2.2 μM Av_2.

nitrogenase activity (electron transfer to substrates) decreases more rapidly than the ATPase activity. This means that in contrast to Fe protein, partial inactivation of MoFe protein can uncouple the ATPase activity of the complex significantly from substrate reduction. This result is in agreement with the observations of Rennie et al. [22]. They found that in case of inhibition of nitrogenase activity with antibodies raised against Fe protein substrate reduction and ATPase activity were inhibited to the same extent, while antibodies raised against MoFe protein inhibited substrate reduction more than ATPase activity.

Fig. 1 also shows that the reductant-independent ATPase activity of nitrogenase is not as O_2-sensitive as the substrate reducing activity. Up to an inactivation of at least 50% of the electron-transfer activity, there is no effect upon the reductant-independent ATPase activity. When the substrate reducing activity of the nitrogenase complex was zero due to inactivation by O_2 of either Av_1 or Av_2, there still remained a residual reductant-independent ATPase activity of about 20% of the maximal activity under the given experimental conditions.

The nature of the reductant-independent ATPase activity is still unclear. It has been suggested that reductant-independent ATPase activity originates from a futile cycle. Electrons present on the EPR active form of MoFe protein are transferred back to Fe protein and then from Fe protein coupled to MgATP hydrolysis to the oxidized form of MoFe protein (EPR silent). Our investigations show that after dye-oxidation of the separate components, the recombined nitrogenase complex has the same reductant-independent ATPase activity as compared to the ATPase activity of a nitrogenase complex that due to prolonged catalysis has run out of $Na_2S_2O_4$ (results not shown). Evidence that the reductant-independent ATP hydrolysis is not obligatory coupled to electron transfer from Fe protein to MoFe protein was previously given by demonstrating rapid pre-steady-state MgATP hydrolysis by dye-oxidized nitrogenase proteins [23].
These two observations indicate that the reductant-independent ATPase activity is not coupled at all to electron transfer from Fe protein to MoFe protein, but is an intrinsic activity of the complex between the nitrogenase proteins.

Saturation Curves for MoFe protein and Fe protein.

Since our results show that futile electron cycling cannot be the explanation for the nature of the reductant-independent ATPase activity we tried to get more insight in the role of Fe protein and MoFe protein in the reductant-independent ATPase activity by titration assays. The reductant-independent ATPase activity was compared with the reductant-dependent ATPase activity (Fig. 2). Addition of a low amount of Av_1 to Av_2 results in a rapid increase in reductant-independent ATPase activity expressed per mg Av_2 (Fig. 2A). While the maximal reductant-dependent ATPase activity (4700 nmol ADP.min^{-1}.$mg^{-1}Av_2$) is reached at a molar ratio of Av_1/Av_2 above 2, the maximal reductant-independent ATPase activity (560 nmol ADP.min^{-1}.$mg^{-1}Av_2$) is reached already at Av_1/Av_2 ratios above 0.5. The maximal turnover numbers for the ATPase activity of Av_2 in the absence and presence of reductant are 0.3 s^{-1} and 4.8 s^{-1} respectively, assuming the hydrolysis of 2 molecules of MgATP per Av_2 turnover. Fig. 2B shows the effect of excess Av_2 over Av_1 upon the ATPase activity (expressed per mg Av_1) plus or minus reductant. A gradual rise in activity was found with increasing amounts of Av_2 and at higher ratios the increase in activity levels off. Hill plots of the ATPase activity titration data of Fig. 2B show linearity over a wide range of velocities (not shown). The Hill coefficients of 1.05 and 1.06 indicate that there is no cooperative interaction between the sites for Av_2 on Av_1. The data points of Fig. 2B were fitted to hyperboles. Extrapolation of these curves to saturating concentrations of Fe protein yields maximal ATPase activities of 5676 and 5000 nmol ADP.min^{-1}.mg^{-1} Av_1 in the presence and absence of reductant, respectively. The calculated turnover numbers are 5.2 s^{-1} and 4.6 s^{-1} for the reductant-dependent and reductant-independent ATPase activity of Av_1, assuming the hydrolysis of 4 molecules of MgATP per Av_1 turnover.

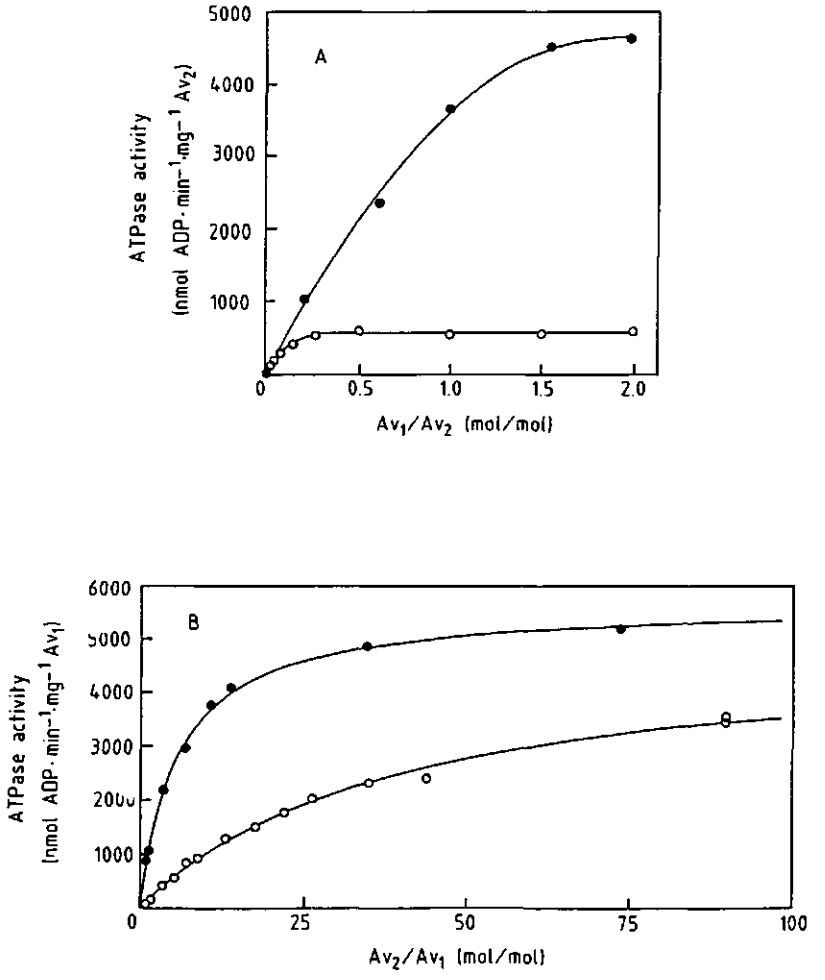

Fig. 2. **Dependence of the reductant-dependent and reductant-independent ATPase activity on the ratio Av_1:Av_2.**

Experimental conditions are described in Materials & Methods. (A) Plot of nmol ADP formed $min^{-1}.mg^{-1}$ Av_2 versus component ratio. Each assay contained 1 µM Av_2 and in case of reductant-dependent ATPase activity 0.2 mM flavodoxin was added to the assay mixture in addition to $Na_2S_2O_4$. (B) Plot of nmol ADP formed $min^{-1}.mg^{-1}.Av_1$ versus component ratio. Each assay contained 0.5 µM Av_1 and varying concentrations of Av_2 as indicated. Closed symbols are reduced proteins, open symbols are oxidized proteins. The molar component ratio is calculated on the basis of relative molecular mass values of 220 kDa and 63 kDa for Av_1 and Av_2, respectively.

Variation of Reductant-independent ATPase Activity with Temperature and pH.

Data about the temperature dependence of the rate of ATP hydrolysis by the nitrogenase complex are only reported for nitrogenase in the presence of dithionite [24,25]. We examined the effect of the temperature upon the reductant-independent ATPase activity at 22°C and 30°C. A temperature increase from 22°C to 30°C leads to a 35% increase in the reductant-independent ATPase activity, while a twofold increase of the reductant-dependent ATPase activity was observed.

The pH dependence of the reductant-independent ATPase activity was examined for Cp nitrogenase [4] and Kp nitrogenase [6]. We found for a 1:2 mixture of Av_1 and Av_2 a gradual increase in ATPase activity when the pH was lowered from pH 8.0 (270 nmol P_i/min.mg Av_2) to pH 5.8 (900 nmol P_i/min.mg Av_2). Below pH 5.8 there was a decrease in ATPase activity, probably due to enzyme inactivation.

The Protein 'Dilution Effect' on Reductant-independent ATPase Activity

The non-linear decline of the enzyme activity when the protein concentration in the assay is decreased at a constant ratio of the nitrogenase proteins ("dilution effect") was used by Thorneley et al. [26] to determine the dissociation constant of the nitrogenase complex. It was assumed that substrate reduction activity only was associated with the complexed proteins. The dilution effect was explained as a mass action effect on the equilibrium of free and complexed proteins, assuming existing thermodynamic equilibrium during steady-state catalysis. Analysis of such data yielded association constants of 2.0×10^7 M^{-1} and 1.4×10^7 M^{-1} for the nitrogenases of Kp and Ac, respectively. As discussed later (Thorneley and Lowe, [27]) the dilution effect might also be due to the fact that thermodynamic equilibrium between components and complex does not exist during catalysis. Our measurements of the reductant-independent ATPase activity at low protein concentrations of different mixtures (1:1, 1:4 and 1:22) of oxidized Av_1 and Av_2 yielded activity versus protein concentration curves comparable to those reported by Thorneley et al. [26] for nitrogenase activity of Kp and Ac (not shown). From these curves an association constant of about 5×10^6 M^{-1} was estimated. This indicates that the dilution phenomena observed with the oxidized nitrogenase proteins are comparable with those of the reduced nitrogenase proteins.

MgATP Dependence of Reductant-independent ATPase Activity.

Information about the role of MgATP in nitrogenase catalysis has come from kinetic studies. From sigmoidal kinetics, Hageman et al. [28] suggested that two molecules of MgATP must be bound to nitrogenase before MgATP is hydrolyzed. We present here steady-state kinetic data on the MgATP dependence of the reductant-independent ATPase activity of nitrogenase and these data are compared with those measured for the reductant-dependent ATPase activity (Fig. 3). The MgATP dependence of the reductant-independent

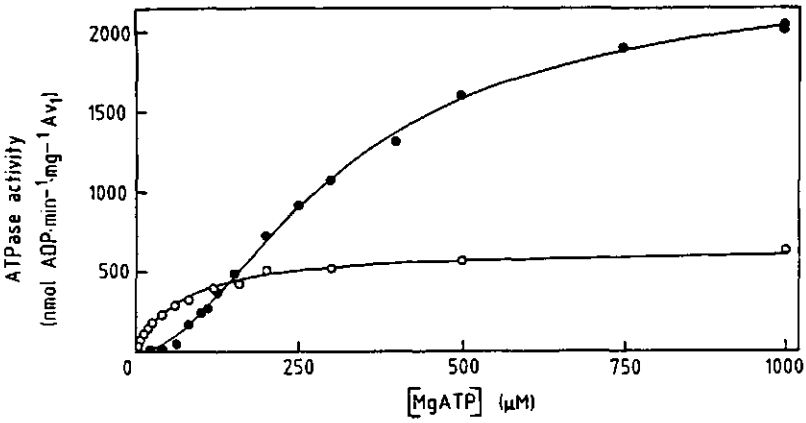

Fig. 3. **Dependence of the reductant-independent and the reductant-dependent ATPase activity on the concentration of MgATP.**
Assay conditions are as described in Materials & Methods except that the concentration of MgATP was varied as shown. Closed symbols are reduced proteins, open symbols are oxidized proteins. (●-●), 0.5 μM Av_1 and 1.5 μM Av_2; (o-o), 0.5 μM Av_1 and 2 μM Av_2.

ATPase activity differs in two essential ways from that of the reductant-dependent ATPase activity (or substrate reduction). Firstly, the reductant-dependent ATPase reaction shows a sigmoidal dependence on MgATP concentration; in the MgATP-dependence of the reductant-independent ATPase reaction such sigmoidal MgATP kinetic pattern is not apparent and if existing it must be close to the origin (see Fig. 3).

Secondly, in contrast to reductant-dependent MgATP hydrolysis, the K_m-value for MgATP of reductant-independent MgATP hydrolysis is dependent on the ratio of Av_1 and Av_2 (see Table 1). The K_m-value for MgATP of reductant-independent ATPase at higher ratios of Av_2/Av_1 approaches the value of the dissociation constant of MgATP for oxidized Av_2 [9]. The kinetic properties of the reduced protein are complicated. The positive cooperativity with respect to activity and the negative cooperativity with respect to the binding of MgATP to reduced Av_2 [9] will be discussed in Chapter 6.

Table 1: MgATP dependence of the reductant-independent ATPase activity at different ratios of the nitrogenase proteins.

Ratio Av_2/Av_1	K_m (μM)	V_{max} (nmol ADP.min^{-1}.mg^{-1} Av_1)
1	12.0	170
4	69.5	741
90	123.7	3453

5.5. Discussion

Reductant-independent ATPase activity was measured in assay mixtures exhausted of dithionite due to nitrogenase activity [1,5,6]. The residual low rate of MgATP hydrolysis observed after exhaustion of reductant was attributed to a process called futile cycling. This hypothetical futile cycling of electrons involves the transfer of an electron from Fe protein to MoFe protein coupled to the hydrolysis of MgATP, followed by the re-reduction of the Fe protein by MoFe protein. Our data demonstrate that steady-state MgATP hydrolysis also occurs when all clusters which normally are involved in the electron transfer reactions, are oxidized. Since the steady-state ATPase activity of the dye-oxidized nitrogenase proteins of A.vinelandii shows the same features as published previously for the reductant-independent ATPase activity of nitrogenase from K.pneumoniae and C.pasteurianum, it is concluded that the reductant-independent ATPase acti-

vity of nitrogenase is a property of the oxidized proteins, and not the result of futile cycling of electrons.

In this paper steady-state kinetic data are presented for the ATPase activity of nitrogenase in the presence and in the absence of reductant. These data have been used to formulate a mechanism for nitrogenase action, based on the current models for the coupling of electron transfer between the nitrogenase proteins and MgATP hydrolysis. Until now only models are reported that describe the kinetics of the electron transfer reactions of nitrogenase. By developing a mechanism that describes the kinetics of both the reductant-dependent and the reductant-independent ATPase activity, more insight in the functioning of the Fe protein during nitrogenase catalysis can be obtained. Fig. 4 gives a schematic presentation of the two kinetic models that have been constructed to simulate the experimental data (models I and II). The derivation of the rate equations for these two models is given in the Appendix. To test whether the rate equation of such a model fits the data points, calculations have been performed in which the values of the rate constants that are known from the literature, have been substituted as such in the mathematical formulation of the rate equation. Since not all rate constants of the reactions used in the models I and II have been measured, assumptions of some of these constants must be made. The mechanistic models I and II should be capable of simulating the kinetic patterns of, firstly, the titration curves of Fig. 2 and, secondly, the MgATP dependence of the ATPase activity (Fig. 3).

Recently it has been shown that the pre-steady-state burst of MgATP hydrolysis is not inhibited or abolished by oxidation of the proteins [23]. This indicates that the rate constants of MgATP hydrolysis by oxidized and reduced proteins have approximately the same values i.e. $k_7 \approx 200\ s^{-1}$. Thus the difference in the steady-state rates of reductant-dependent and reductant-independent ATPase activity cannot be explained by a difference in the rate constant of MgATP hydrolysis. Thorneley and Lowe [29] concluded from pre-steady-state experiments and steady-state experiments that the rate-limiting step in the catalytic cycle of nitrogenase during substrate reduction is the dissociation of oxidized Fe protein from MoFe protein after MgATP induced electron transfer has occurred (k_9). This means that the rate-limiting step during reductant-dependent ATPase activity occurs after the hydrolysis of MgATP.

In case of reductant-independent ATPase activity the rate-limiting step might take place before the hydrolysis of MgATP by the complex (see model I).

Since pre-steady-state experiments [23] have shown that the association rate of oxidized nitrogenase complex with MgATP (k_3) is fast and comparable with that of reduced nitrogenase complex, this reaction can be excluded as being different under both conditions. However, by adjusting the value of the rate of dissociation (k_{14}) of Av_1 from the $Av_1.Av_2.MgATP$ complex of

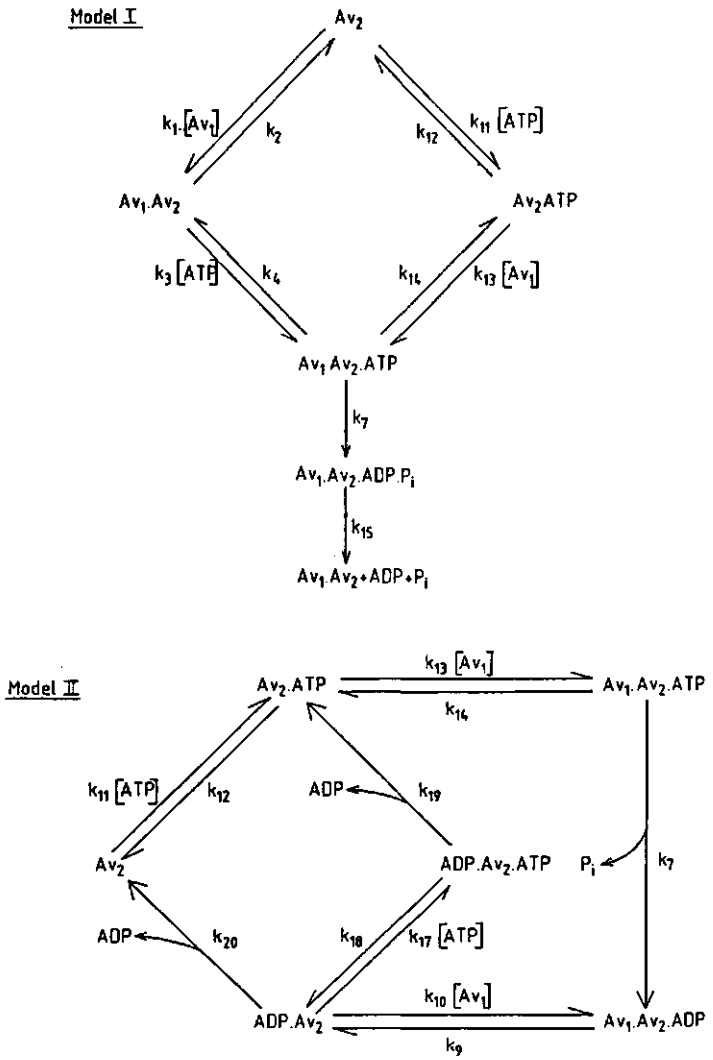

Fig. 4. Models used to describe the reductant-independent and the reductant-dependent ATPase activity of nitrogenase.
The numbering of the rate constants is the same for the models presented in Chapters 5 and 6 of this thesis.

Table 2. **Comparison of the experimentally determined ATPase activity of Av_1 with the ATPase activity as calculated from the rate equations derived according to the models I and II.**
For model I the velocity was calculated using equation 1 (see Appendix). The rate constant k_{14} for model I was determined by minimalizing equation 1. For model II the rate constants k_{19} and k_{20} were determined by minimalizing equation 4. Both minimalizations were performed by using the experimentally determined activity of a mixture of 0.2 μM Av_1, 1 μM Av_2 and 5 mM ATP with or without 20 mM $Na_2S_2O_4$.
The known variables (rate constants, protein and ATP concentrations) were used to calculate the velocity. The calculated velocity (v_{calc}) was compared with the experimentally determined velocity (v_{exp}) for an Av_1/Av_2 ratio of 1:100, and in case of model I also for a low concentration of MgATP.
The rate constants used in the calculations were taken from Lowe and Thorneley [29] and from Mortenson and Thorneley [30].
k_1 = 5 x 10^7 $M^{-1}.s^{-1}$; k_2 = 15 s^{-1}; k_{11} = 1 x 10^7 $M^{-1}.s^{-1}$; k_{12} for oxidized proteins = 1 x 10^3 s^{-1}; k_{12} for reduced proteins = 4 x 10^3 s^{-1}; k_3 for oxidized proteins = 1 x 10^7 $M^{-1}.s^{-1}$; k_3 for reduced proteins = 2.5 x 10^6 $M^{-1}.s^{-1}$; k_4 = 1 x 10^3 s^{-1}; k_{13} = 5 x 10^7 $M^{-1}.s^{-1}$; k_{14} variable in model I; in model II k_{14} = k_2; k_7 = 200 s^{-1}; k_9 = 6.4 s^{-1}; k_{10} = 4.4 x 10^6 $M^{-1}.s^{-1}$; k_{15} = 200 s^{-1}; k_{17} = k_{11}; k_{18} = k_{12} ; k_{19} for oxidized proteins = 0.8 s^{-1}; k_{19} for reduced proteins = 5.0 s^{-1}; k_{20} = 1 x 10^{-3} s^{-1}.

Model	Protein	Rate constants used in the calculation	[MgATP] (M)	v_{exp} (nmol ADP.min^{-1} $mg^{-1}Av_1$)	v_{calc} (nmol ADP.min^{-1} $mg^{-1}Av_1$)
I	0.2 μM Av_1(ox) 1.0 μM Av_2(ox)	k_{14} = 4.4 x 10^3 s^{-1}	5x10^{-3} 1x10^{-4}	266 169	268 115
	0.1 μM Av_1(ox) 10 μM Av_2(ox)		5x10^{-3}	1760	991
I	0.2 μM Av_1 1.0 μM Av_2(red)	k_{14} = 7 x 10^2 s^{-1}	5x10^{-3} 0.3x10^{-3}	1290 645	1289 684
	0.1 μM Av_1 10 μM Av_2(red)		5x10^{-3}	2678	2335
II	0.2 μM Av_1(ox) 1.0 μM Av_2(ox)	k_{19} = 0.8 s^{-1}	5x10^{-3}	266	264
	0.1 μM Av_1(ox) 10 μM Av_2(ox)		5x10^{-3}	1760	1408
II	0.2 μM Av_1 1.0 μM Av_2(red)	k_{19} = 5.0 s^{-1}	5x10^{-3}	1290	1240
	0.1 μM Av_1 10 μM Av_2(red)		5x10^{-3}	2678	2575

oxidized or reduced proteins, the calculated rates (see Appendix, Eqn. 1) of the reductant-dependent and reductant-independent ATPase activity (at a ratio of Av_1 and Av_2 = 1:5) can be brought in agreement with the experimentally determined rates (Table 2).

As can be seen from the rate equations (see Appendix) model I predicts a *sigmoidal dependence of the velocity on the concentration of MgATP and* also predicts that the K_m-value for MgATP is different for different ratios of Av_1 and Av_2.

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As can be seen from the rate equations (see Appendix) model I predicts a sigmoidal dependence of the velocity on the concentration of MgATP and also predicts that the K_m-value for MgATP is different for different ratios of Av_1 and Av_2.

Model II represents another mechanism developed to explain the titration curves obtained for reductant-dependent and reductant-independent ATPase activity of nitrogenase. In the model the rate-limiting steps occur after the hydrolysis of MgATP by the complex (k_7), that is either the release of MgADP from Av_2.MgADP (k_{20}) or from Av_2.MgADP.MgATP (k_{19}). Table 2 shows that when it is assumed that all values of the rate constants in model II are identical for reductant-dependent and reductant-independent ATPase activity, except the values for K_b (k_{18}/k_{19}), K_d (k_{12}/k_{11}) and k_{19}, then model II follows the titration curves of both ATPase activities just as model I. As can be seen in Table 2, the rate constant with the largest contribution to the overall steady-state rate of MgATP hydrolysis under both conditions is k_{19}, the rate of dissociation of MgADP from the Av_2.MgADP.MgATP complex. k_{19} differs by a factor 6 between reductant-dependent and reductant-independent ATPase activity. The explanation for the difference in the k_{19} value might be that reduction of Av_2(ox) MgADP.MgATP results in a higher rate of dissociation of MgADP from Av_2.MgADP.MgATP. Model II cannot predict the different kinetic patterns obtained for the reductant-dependent and reductant-independent ATPase acti-

cific ATPase activity of Av_1 under reductant-dependent and reductant-independent ATPase conditions. In addition model I predicts sigmoidal dependence of the ATPase activity on the MgATP concentration. Indications for the slow dissociation of MgADP from Av_2 MgADP ($k_{20} = 1x10^{-3}\ s^{-1}$) is tight-binding of one molecule of MgADP to Av_2 during nitrogenase catalysis. For a more rigorous quantitative understanding of nitrogenase action, the estimation of more rate constants used in the models is required.-

Appendix

The rate equation for model I (Fig. 4) is derived from the steady-state rate equations of the separate reaction steps by assuming that $[ADP] = [P_i] = 0$ and $[Av_2] \approx [Av_2]_o$, where $[Av_2]_o$ is the total concentration of Av_2. The expression for v then becomes:

$$v = \frac{\frac{k_{15}k_7}{k_{15}+k_7}[Av_1]_o}{1+\frac{(k_1[Av_2]+k_2)(k_4+k_7+k_{14})k_{15}+(k_4+k_7)k_{13}k_{15}K_a[ATP][Av_2]+k_3+k_{14}k_{15}[ATP]}{(k_{15}+k_7)\{k_1k_3[Av_2][ATP]+(k_2+k_3[ATP])k_{13}K_a[ATP][Av_2]}} \quad (1)$$

in which $K_a = \frac{k_{11}}{k_{12}}$ is the association constant of Av_2.ATP.

Rearrangement of Eqn. (1) with the object to eliminate all terms only dependent on Av_2 leads to:

$$v = \frac{V_m[Av_2][ATP](k_7+k_{15})\{k_1k_3+(k_2+k_3[ATP]k_{13}K_a\}}{[Av_2][ATP](k_7+k_{15})\{k_1k_3+(k_2+k_3[ATP]k_{13}K_a\}+(k_1[Av_2]+k_2)(k_4+k_7+k_{14})k_{15}+k_3k_{14}k_{15}[ATP]} \quad (2$$

$$1+\frac{(k_4+k_7)k_{13}k_{15}K_a}{(k_7+k_{15})\{k_1k_3+(k_2+k_3[ATP])k_{13}K_a\}+\frac{(k_1[Av_2]+k_2)(k_4+k_7+k_{14})k_{15}}{[Av_2][ATP]}+\frac{k_3k_4k_{15}}{[Av_2]}}$$

At not saturating ATP concentrations the terms

$\frac{(k_1[Av_2] + k_2(k_4 + k_7 + k_{14})k_{15}}{[Av_2][ATP]}$ and $\frac{k_3k_4k_{15}}{[Av_2]}$ contribute to the apparent K_m-value for ATP. As can be seen these terms are dependent on the Av_2 concentrations. This might explain the observed dependency of the K_m-value for ATP on the ratio of Av_1 and Av_2 (see Table 1).

The same procedure can be followed to find the kinetic constants that might deter-

mine the difference of the K_m-value for Av_2 of the reductant-dependent and reductant-independent ATPase activity of Av_1.
Eqn. (1) can be rearranged to Eqn. (3):

$$v = \frac{\dfrac{V_m(k_{15}+k_7)\{k_1k_3+(k_2+k_3[ATP])k_{13}K_a\}}{(k_{15}+k_7)\{k_1k_3+(k_2+k_3[ATP])k_{13}K_a+(k_4+k_7)k_{15}k_{13}K_a}}{1+\dfrac{(k_1[Av_2]+k_2)(k_4+k_7+k_{14})k_{15}+k_3k_{14}k_{15}[ATP]}{[Av_2][ATP](k_{15}+k_7)\{k_1k_3+(k_2+k_3[ATP]k_{13}K_a\}+(k_4+k_7)k_{13}k_{15}K_a}} \qquad (3)$$

If [ATP] → ∞, all terms with ATP become dominant, so that K_m (Av_2) becomes:

$$\frac{k_{14}k_{15}}{(k_{15}+k_7)k_{13}K_A[ATP]}$$

Thus the difference in K_m-value for Av_2 of reductant-dependent and reductant-independent ATPase activity might be explained by a difference in the rate constants k_{13} and k_{14}, i.e. in case of oxidized proteins k_{14} must be larger (or k_{13} smaller) than in case of reduced proteins. The observed smaller K_a-value for oxidized proteins compared with reduced proteins stimulates the effect. The value of k_{14} was adjusted to fit Eqn. (1) to the data points of Fig. 2.

Elimination of the enzyme intermediates present in the separate rate equations of model II yields an overall equation that contains in addition to the different rate constants, the variables v, [ATP], $[Av_1]_o$ and $[Av_2]_o$:

$$C_1+C_2+k_9C_2/k_7+k_{10}C_1C_3/k_9+\frac{k_9(k_7+k_{14})C_2}{k_7k_{13}C_3}+C_1[ATP]/K_b+$$

$$+\frac{k_9(k_7+k_{14})K_dC_2}{k_7k_{13}C_3[ATP]}-[Av_2]_o=0 \qquad (4)$$

in which

$$C_1=\frac{vK_b}{k_{20}+k_{19}[ATP]}$$

$$C_2=k_{20}C_1/k_9$$

$$C_3 = \frac{[Av_1]_o - k_9C_2/k_7 - C_2}{1 + k_{10}C_1/k_9}$$

$$K_b = \frac{k_{18}}{k_{17}} \text{ and } K_d = \frac{k_{12}}{k_{11}}$$

Since it is mathematically not easy to solve v from equation 4 (the equation has the expression of $av^3 + bv^2 + cv + d = 0$) it was decided to use equation 4 direct in the calculations to verify if model II could describe the titration curves. Eqn. (4) was used to determine a value for k_{19} and k_{20} to fit the activity of a mixture of 0.2 μM Av_1, 1 μM Av_2 and 5 mM ATP in the presence of 20 mM $Na_2S_2O_4$ (reduced proteins) or without addition of $Na_2S_2O_4$ (oxidized proteins). At other protein concentrations the velocity was calculated with the same rate constants by adjusting the velocity to fit equation 4.

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References

1. Ljones, T. & Burris, R.H. (1972) Biochim.Biophys.Acta 275, 93-101.
2. Bui, P.T. & Mortenson, L.E. (1969) Biochemistry 8, 2462,2465.
3. Hadfield, K.L. & Bulen, W.A. (1969) Biochemistry 8, 5103-5108.
4. Jeng, D.Y., Morris, J.A. & Mortenson, L.E. (1970) J.Biol.Chem. 245, 2809-2813.
5. Mortenson, L.E., Walker, M.N. & Walker, G.A. (1976) in Proceedings of the 1st International Symposium on Nitrogen Fixation, vol. 1 (Newton, W.E. & Nijman, C.J., eds.) pp. 117-149, Washington State University Press.
6. Iman, S. & Eady, R.R. (1980) FEBS Lett. 110, 35-38.
7. Orme-Johnson, W.H. & Davis, L.C. (1977) in Iron Sulfur Proteins (Lovenberg, W., ed.) pp. 15-60, New York: Academic Press Inc., New York.
8. Cordewener, J., Haaker, H. & Veeger, C. (1983) Eur.J.Biochem. 132, 47-54.
9. Cordewener, J., Haaker, H., Van Ewijk, P. & Veeger, C. (1985) Eur.J.Biochem. 148, 499-508.
10. Johnson, M.K., Thomson, A.J., Robinson, A.E. & Smith, B.E. (1981) Biochim.Biophys.Acta, 671, 61-70.
11. Braaksma, A., Haaker, H., Grande, H.J. & Veeger, C. (1982) Eur.J.Biochem. 12, 483-491.
12. Ennor, A.H. (1957) in Methods in Enzymology (Colowick, S.P. & Kaplan, N.O., eds.) pp. 850-861, Academic Press Inc., New York.
13. Bensadoun, A. & Weinstein, D. (1976) Anal.Biochem. 70, 241-250.
14. Goa, J. (1953) Scand.J.Clin.Lab.Invest. 5, 218-222.
15. Ottolenghi, P. (1975) Biochem.J. 151, 61-66.
16. Baginski, E.S., Foa, P.P. & Zak, B. (1967) Clin.Chim.Acta 15, 155-158.
17. Hadfield, K.L. & Bulen, W.A. (1969) Biochemistry 8, 5103-5108.
18. Silverstein, R. & Bulen, W.A. (1970) Biochemistry 9, 3809-3815.
19. Watt, G.D., Bulen, W.A., Burns, A. & Hadfield, K.L. (1975) Biochemistry 14, 4266-4272.
20. Hagemann, R.V. & Burris, R.H. (1978) Biochemistry 17, 4117-4124.
21. Emerich, D.W. & Burris, R.H. (1976) Proc.Natl.Acad.Sci.U.S.A. 73, 4369-4373.
22. Rennie, R.J., Funnel, A. & Smith, B.E. (1978) FEBS Lett. 91, 158-161.
23. Cordewener, J., ten Asbroek, A., Wassink, H., Eady, R., Haaker, H. &

Veeger, C. (1987) Eur.J.Biochem., 162, 265-270.
24. Burns, R.C. (1969) Biochem.Biophys.Acta 171, 253.
25. Watt, G.D. & Burns, A. (1977) Biochemistry 16, 264-270.
26. Thorneley, R.N.F., Eady, R.R. & Yates, G. (1975) Biochim.Biophys.Acta 403, 269-283.
27. Thorneley, R.N.F. & Lowe, D.J. (1984) Biochem.J. 224, 903-909.
28. Hageman, R.V., Orme-Johnson, W.H. & Burris, R.H. (1980) Biochemistry 19, 2333-2342.
29. Lowe, D.J. & Thorneley, R.N.F. (1984) Biochem.J. 224, 877-886.
30. Mortenson, L.E. & Thorneley, R.N.F. (1979) Ann.Rev.Biochem. 48, 387-418.

Chapter 6
General discussion

6.1. Adenine nucleotide binding properties of the separate nitrogenase proteins.

Since 1965 [1] it is known that during the nitrogenase catalysis reducing equivalents and MgATP are both necessary. To obtain more insight in the involvement of MgATP in nitrogenase catalysis, adenine nucleotide binding studies on the separate components were performed. Bui and Mortenson [2] showed that Cp_2, but not Cp_1, binds MgATP and MgADP. Quantitative results obtained from direct binding studies are only available for Cp_2 [3] and Kp_1 [4]. Tso and Burris [3] showed that reduced Cp_2 has two binding sites for MgATP and one for MgADP. No binding of MgATP or MgADP to Cp_1 could be demonstrated [2,3], but controversially later it was reported that Kp_1 binds 4 molecules of MgATP [4].

In Chapters 2 and 3 of this thesis a comprehensive study is given of the binding of MgATP and MgADP to both oxidized and reduced Av_2. The binding properties of Av_2 for MgATP and MgADP were found to be dependent on two factors: the specific activity of the protein preparation used and the oxidation state of the protein. In the presence of 1 mM MgATP reduced Av_2 binds about 1 mol MgATP/mol Av_2 (K_d = 0.15 mM), while oxidized Av_2 binds under similar conditions 2 mol MgATP/mol Av_2 (K_1 = 0.049 mM, K_2 = 0.18 mM). At a free concentration of MgADP of 1 mM, both oxidized and reduced Av_2 bind 2 mol MgADP/mol Av_2. MgADP has a higher affinity for oxidized Av_2 (K_1 = 0.024 mM, K_2 = 0.039 mM) than for reduced Av_2 (K_1 = 0.091 mM, K_2 = 0.044 mM). Furthermore, the binding of MgADP to reduced Av_2 shows positive cooperativity.

In competition experiments with MgATP and MgADP, and also from gel filtration experiments it was found that a maximal amount of two adenine nucleotides (MgATP plus MgADP) could be bound to Av_2. It was concluded that MgATP and MgADP compete for the same binding sites on Av_2. This means that also in case of reduced Av_2 two MgATP binding sites are present on the protein. However, when one binding site for MgATP on the Fe protein is occupied, due to strong negative cooperativity the second adenine nucleotide binding site has a low affinity for MgATP. The K_d for binding of a second molecule of MgATP to reduced Av_2 is approximately 1.7 mM.
The binding of MgADP to oxidized and reduced Av_2 is much stronger than the binding of MgATP. These results are in agreement with the competition

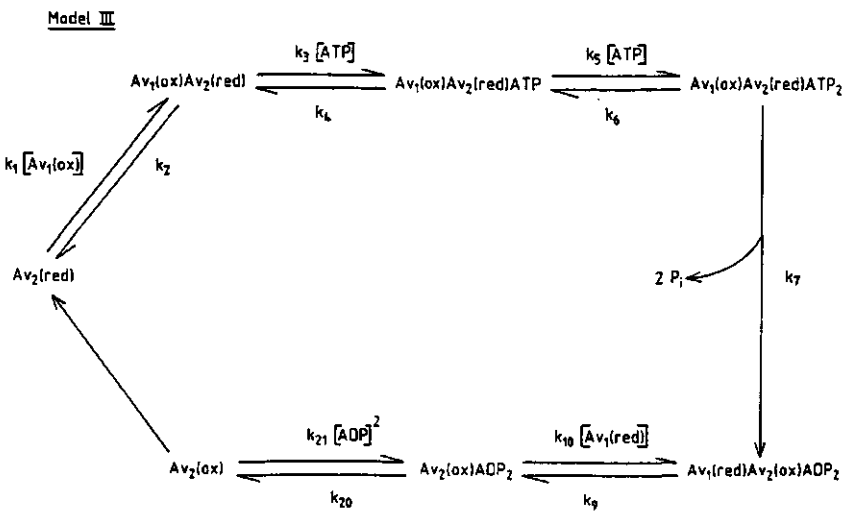
experiments of Chapter 3 where it was shown that a relatively low concentration of MgADP results in a strong inhibition of the binding of MgATP to Av_2. These binding data explain the strong inhibitory effect of MgADP on nitrogenase activity.

6.2. Adenine nucleotide binding properties of the nitrogenase complex.

Due to the ATPase activity of nitrogenase direct binding studies of MgATP to the complex are impossible. To obtain information about the interaction of MgATP with the complex one can perform kinetic measurements. The MgATP dependence of the overall nitrogenase reaction has been studied and also that of the pre-steady-state electron transfer from Fe protein to MoFe protein. Both reactions show a sigmoidal dependence on MgATP concentration. This type of kinetic behaviour was explained by Hageman et al. [5] by assuming that the nitrogenase reaction requires the simultaneous binding of two molecules of MgATP to the complex before MgATP hydrolysis and electron transfer occurs. The general equation for such a reaction mechanism is:

$$v = \frac{V_{max}[MgATP]^2}{K_A K_B + K_B[MgATP] + [MgATP]^2} \qquad (1)$$

in which K_A and K_B are composed of different rate constants. Dependent on the reaction rate (v) that is measured K_A and K_B have different values (see model III). For instance, when the dependence of H_2 evolution on MgATP concentration is measured, the equations for K_A and K_B are:



$$K_A = \frac{k_4}{k_3} \qquad (2)$$

$$K_B = \frac{(k_6 + k_7)k_9}{k_5 k_7} \qquad (3)$$

A numerical estimate for the different rate constants under the given experimental conditions can be made. An estimation of k_6 can be made by using the value of K_B = 0.97 mM as determined by Hageman et al. [5] for the MgATP dependence of the pre-steady-state electron transfer reaction between Av_2 and Av_1. Substituting for K_B = 0.97 mM, for k_6/k_5 = 0.85 mM (intrinsic dissociation constant for binding of the second MgATP to reduced Av_2, see Chapter 3) and for k_7 = 200 s^{-1} [cf. ref. 7] into equation 4 that determines K_B for the pre-steady-state ATP hydrolysis

$$K'_B = \frac{k_6 + k_7}{k_5} \qquad (4)$$

gives the values of k_6 = 1.4 x 10^3 s^{-1} and k_5 = 1.7 x 10^6 $M^{-1}s^{-1}$. Thus k_6 and k_7 differ by a factor 7. This means that equation 3 (K_B for H_2 production) reduces to:

$$K_B \approx \frac{k_6 k_9}{k_5 k_7}$$

The maximal turnover number observed for nitrogenase at 23°C and pH 7.4 is k_9 = 6.4 s^{-1} [6], while k_7 has a value of 200 s^{-1} [7]. The ratios of the rate constants k_4/k_3 and k_6/k_5 are equal to the dissociation constants of the first respectively second molecule of MgATP that binds to the complex of Av_1 and reduced Av_2. When the dissociation constants for the binding of MgATP to reduced Av_2 as reported in Chapter 3 (K_1^i = 0.44 mM, K_2^i = 0.85 mM) are substituted in Eqns. (2) and (3), the kinetic constants for the H_2 evolution reaction become: K_A = 0.44 mM and K_B = 0.027 mM. These values are remarkably close to the values reported by Hageman et al. [5] for the H_2 evolution reaction, namely K_A = 0.39 mM and K_B = 0.030 mM. This means that if $k_6 >> 200$ s^{-1}, the sigmoidal MgATP dependence of the H_2 evolution reaction can be explained by using our dissociation constants of MgATP to reduced Av_2 showing negative cooperativity. In other words, kinetic model III cannot be used to support the correctness of the dissociation constants as reported by Hageman et al. [5] for the binding of MgATP to free, reduced Av_2.

While the MgATP dependence of the reductant-dependent ATPase activity

shows sigmoidal kinetics, the MgATP dependence of the ATPase activity of different ratios of oxidized Av_1 and oxidized Av_2 yields hyperbolic curves (see Chapter 5). The data points of the reductant-independent ATPase activity can be fitted with the Michaelis-Menten equation, using different K_m-values depending upon the ratio of the proteins. This phenomenon has been explained in the Appendix of Chapter 5. It indicates that in case the complex of oxidized Av_1 and Av_2 has two binding sites for MgATP these binding sites are identical and independent. This is in agreement with the binding studies of MgATP to free, oxidized Av_2, showing the binding of two molecules of MgATP with the same intrinsic dissociation constant K^i = 95 ± 29 μM. In other words model III only explains the kinetic behaviour of the oxidized proteins, in case $K_A << K_B$ which means $K_A \approx 1$ μM in contrast to the value actually determined. For this reason we have introduced models I and II in Chapter 5.

From stopped-flow experiments Thorneley and Cornish-Bowden [8] concluded that MgADP is a competitive inhibitor of MgATP during MgATP dependent electron transfer from Kp_2 to Kp_1. They reported a dissociation constant K_i^1 = 0.020 mM for the binding of MgADP to the complex of Kp_1 and Kp_2 during pre-steady-state electron transfer between the two proteins. Our data for the binding of MgADP to the complex of Av_1 with reduced Av_2 yielded two dissociation constants, namely K_1^i = 0.18 mM and K_2^i = 0.022 mM. Besides assuming that there are species differences, these apparently conflicting results can be explained by assuming that from the two MgADP binding sites present on the nitrogenase complex only the one with the highest affinity for MgADP is of importance for the MgATP dependent electron transfer reaction.

6.3. ATPase activity of nitrogenase.

6.3.1. **Electron transfer cycle.**

Model III represents a possible mechanism for the coupling between the electron transfer between the nitrogenase proteins and MgATP hydrolysis. Hageman et al. [5] and the Sussex group [6,9] use a similar model to explain most of their experimental results. In the scheme Av_1 represents a $\alpha\beta$-dimer of MoFe protein, containing one FeMo cofactor (FeMoCo) as the active centre, and Av_2 represents dimeric Fe protein. During one cycle one electron is transferred from Fe protein to MoFe protein. Even for the simplest substrate reduction of nitrogenase, the reduction of two protons to H_2, the electron transfer cycle must be completed twice. In pre-steady-

state experiments described in Chapter 4 evidence is given that the maximal stoichiometry of the nitrogenase complex during the pre-steady-state ATPase activity is a 1:2 complex of MoFe protein (2 FeMoCo's) and Fe protein. It is assumed that MoFe protein contains two catalytic centres, and that these two centres are independent. Thus if Fe protein is a one electron donor, the electron transfer cycle must be repeated once before $2H^+$ are reduced. For instance, the reduction of nitrogenase natural substrate, N_2, is achieved by eight sequential cycles of Scheme 1.

The values of most of the rate constants given in model III are now independently determined, mainly by Thorneley and Lowe [6] (see Table 1).

Table 1. **Rate constants as reported by Thorneley and Lowe [6].**

Rate constant	Value
k_1	$5x10^7\ M^{-1}s^{-1}$
k_2	$15\ s^{-1}$
k_7	$200\ s^{-1}$
k_9	$6.4\ s^{-1}$
k_{10}	$4.4x10^6\ M^{-1}s^{-1}$

An important rate constant is k_9, the dissociation of $Fe(ox).MgADP_2$ from MoFe(red). Because this rate constant has a value in the order of the turnover rate of nitrogenase, it is assumed that under optimal conditions the rate of dissociation of the complex is rate-limiting, and not the actual rate of substrate reduction by MoFe protein. Most evidence for the dissociation model III comes from stopped-flow experiments [6], in which the rate of reduction of $Kp_2(ox).MgADP_2$ by $Na_2S_2O_4$ in the presence of Kp_1 was measured. From these kinetic data it was concluded that the rate of reduction of $Kp_2(ox).MgADP_2$ by $Na_2S_2O_4$ when bound to Kp_1 is negligible small. This observation together with the low rate of dissociation of $Kp_2(ox).MgADP_2$ from $Kp_1(red)$ explains why the Fe protein is largely oxidized during steady-state catalysis.

6.3.2. Involvement of MgATP hydrolysis in the electron transfer cycle.

Eady *et al.* [10] observed an identical pre-steady-state time course for both the hydrolysis of MgATP and the electron transfer from Kp_2 to Kp_1.

Hageman et al. [5] have formulated a mechanism for substrate reduction that involves a cycle similar to that of model III. They studied extensively the effect of the concentration of MgATP on the electron transfer rate from Av_2 to Av_1 and on the rate of substrate reduction. From the sigmoidal dependence of the rate of electron transfer on the MgATP concentration it was concluded that two molecules of MgATP are required for the transfer of one electron, consistent with the observed overall stoichiometry of the nitrogenase reaction, i.e. 2 ATP/e. Model III predicts a pre-steady-state reaction in which one electron is transferred and two molecules of MgATP are hydrolyzed. After the electron transfer, the rate-limiting step in nitrogenase turnover occurs, the dissociation of the two proteins. This means that in the pre-steady-state burst a 1:2 complex of MoFe protein and Fe protein hydrolyzes four molecules of MgATP. Hageman et al. [5] found a stoichiometry of 3.2 phosphates produced per Av_1 with a 1:2.5 mixture of Av_1 and Av_2. After corrections made for possible inactive protein, they suggest a stoichiometry of 4.4 phosphates produced per Av_1. The corrections made for inactive protein present in their Av_1 preparation were based on the Mo content (2 Mo per Av_1) of Av_1. However, such a correction of the experimental data is only permitted when it is known that Av_1 lacking Mo atoms does not participate in the pre-steady-state MgATP hydrolysis. The same applies when corrections are made for inactive Fe protein.

Chapter 4 describes pre-steady-state experiments performed to determine the stoichiometry of the P_i-burst at different ratios of Av_1 and Av_2. These experiments show that nitrogenase proteins with a high specific activity (>2000) produce 6-8 molecules of P_i per molecule of Av_1 when ratios of $Av_2/Av_1 \geq 2$ are used. The observed high stoichiometry cannot be explained by model III, which requires for a 1:2 complex a maximal amount of 4 MgATP's hydrolyzed per Av_1 in the burst reaction. The mechanism represented by model III suggests a direct coupling between MgATP hydrolysis and electron transfer. Our pre-steady-state experiments with dye-oxidized nitrogenase proteins (Chapter 4) show a burst reaction similar to that found with reduced nitrogenase proteins, with the same high values of P_i release per Av_1. Thus the initial hydrolysis of MgATP by the nitrogenase complex is independent of electron transfer between the protein components. But this observation gives no explanation for the observed stoichiometry. The maximal value of 8 MgATP's hydrolyzed per Av_1 for a 1:2 complex of Av_1 and Av_2 is puzzling. Since Av_2 has only two adenine nucleotide binding sites and Av_1 no sites at all, this means that if no additional binding sites are

created after complex formation by Av_1 and Av_2, each site has to turnover twice in the pre-steady-state reaction. Since the overall steady-state stoichiometry is 2 MgATP's hydrolyzed per electron transferred from reductant to substrates, this must mean that during or after the pre-steady-state reaction 4 electrons are transferred from reductant via Fe protein to MoFe protein. Our results suggest a mechanism in which MgATP hydrolysis precedes electron transfer. After MgATP hydrolysis the nitrogenase complex is activated for electron transfer. Uncoupling of the steady-state ATPase activity and substrate reduction can be explained with this model by proposing that when after MgATP hydrolysis by the complex the electron transfer is not fast enough, the possibility exists that the complex dissociates and the activation by MgATP hydrolysis is lost.

Chapter 5 deals with the steady-state ATPase activity of nitrogenase in the absence and in the presence of reducing equivalents. The reductant-independent ATPase activity of highly-purified nitrogenases from C.pasteurianum, A.vinelandii and K.pneumoniae were reported to be 5-20% that of the reductant-dependent ATPase activity (pH 7-8; 30°C). To determine the maximal specific ATPase activities of the component proteins that comprise nitrogenase it is necessary to measure the rate of MgATP hydrolysis as a function of the ratio MoFe protein/Fe protein. Therefore the concentration of the protein whose specific activity is being determined is kept constant and the concentration of the complementary protein is varied. When Av_1 was titrated with increasing concentrations of Av_2 (Fig. 2A, Chapter 5), it was observed that the reductant-independent ATPase activity approaches the reductant-dependent ATPase activity at higher ratios of Av_2/Av_1. Assuming the hydrolysis of 8 molecules of MgATP per turnover of Av_1, the turnover rate of Av_1 as calculated from the extrapolated V_{max}-values for the reductant-dependent and reductant-independent ATPase activity are 2.3 s^{-1} respectively 2.1 s^{-1}. So under conditions of excess Av_2 the turnover rate of Av_1 is about the same for the reductant-dependent and the reductant-independent ATPase activity. The same can be done for Av_2 and assuming the hydrolysis of 4 MgATP's per Av_2, the turnover rate of Av_2 is 1.2 s^{-1} and 0.15 s^{-1} for reduced (flavodoxin as electron donor) and oxidized proteins respectively. When dithionite was used as electron donor a maximal turnover rate for Av_2 of 0.8 s^{-1} was measured. From the above-mentioned maximal turnover rates for Av_1 and Av_2 it can be concluded that, firstly, during reductant-dependent ATPase conditions the turnover rate of Av_1 is twice that of Av_2; secondly, during reductant-independent ATPase

conditions the turnover rate of Av_1 is a factor 14 higher than that of Av_2. The 2-fold difference in turnover rate between Av_1 and Av_2 during substrate reduction is in agreement with the observed stoichiometry for the active nitrogenase complex, i.e. an Av_2/Av_1 ratio of 2. Thus this means that the turnover per active site on Av_1 (FeMo cluster) is the same as Av_2 assuming two FeMo clusters per tetramer. An explanation for the much lower turnover rate of Av_2 compared with Av_1 when assayed under reductant-independent ATPase activity conditions, and also when compared with the turnover rates of Av_1 and Av_2 under reductant-dependent ATPase conditions was given in Chapter 5. Although the interpretation of steady-state kinetic data for such a complex multicomponent system as nitrogenase is extremely difficult, we have proposed a model for nitrogenase action under both reductant-dependent and reductant-independent ATPase conditions (see model II, Chapter 5). This model postulates that the rate-limiting step during reductant-independent ATPase activity, and probably also during reductant-dependent ATPase activity (with dithionite as electron donor), is the dissociation of MgADP from Av_2 after MgATP hydrolysis by the complex. This mechanism also prevents MgATP hydrolysis by nitrogenase in the cell as long as there is a shortage of electrons to be used for nitrogen fixation. Although binding experiments described in Chapter 4 indicate a slow dissociation of one molecule of MgADP from Av_2, at the moment there is no direct evidence for such a relatively stable Av_2 intermediate during nitrogenase catalysis. This means that the rate-limiting step given in the model might also be a slow conformational change of Av_2. In any case, the "inactive" form of Av_2 explains why an excess of Av_2 is required for maximal specific ATPase activity of Av_1. The difference in maximal turnover rate of Av_2 during reductant-dependent and reductant-independent ATPase conditions is attributed to a different dissociation rate constant of MgADP from reduced and oxidized Av_2, or a different relaxation time of the "inactive" conformation of reduced and oxidized Av_2.

6.4. Comparison between nitrogenase and ATP synthase.

By comparing the amino acid sequences of the nitrogenase component polypeptides with those of other adenine nucleotide binding proteins, Robson [11] found sequences in Fe protein and in the β-subunit of MoFe protein similar to the α- and β-subunits of E.coli ATP synthase and to the β-subunit of bovine heart mitochondrial ATP synthase. There are more

similarities between both enzyme complexes than those in amino acid sequence and molar masses of the component proteins. Separately Fe protein and the β-subunit of E.coli ATP synthase bind MgATP, but MgATP is only hydrolyzed in the complex [12]. In both cases there is cooperativity in the binding of MgATP. When MgATP binds to Fe protein, there is a large conformational change [13] which might lower, just in case of ATP synthase, the affinity for the binding of a second molecule of MgATP. Also in similarity with the ATP synthase, there is tight binding of adenine nucleotides. In Chapter 4 it was shown that there is a slow dissociation of one molecule of MgATP or MgADP from both reduced and oxidized Av_2. Also when the Fe protein is passed through a gel filtration column (elution time approximately 15 min.) there are still adenine nucleotides bound to the protein: 0.5 mole MgATP or MgADP per mole Av_2(ox) and 0.14 mole MgADP per mole Av_2(red). No binding of MgATP to Av_2(red) was observed under these conditions (J.H. Wassink, personal communication).

Although only isolated Av_2 binds adenine nucleotides, it is still possible that after complex formation of Av_1 and Av_2 additional adenine nucleotide binding sites are generated, consistent with the suggested dual role for MgATP in the mechanism of nitrogenase [14]. For instance, when after the hydrolysis of MgATP at the binding sites on Av_2 through conformational changes MgATP binding sites on Av_1 or, just as in case of the ATP synthase, on the interface between Av_1 and Av_2 are generated, these MgATP binding sites might be very important in substrate reduction. It might be possible that by MgATP hydrolysis at these sites, protons are generated near the substrate binding site which lower the activation energy for reduction of the substrate. It should be realized that for substrate reduction both electrons and protons are necessary. The idea that MgATP binding sites are generated in the nitrogenase complex is supported by the observation that Kp_1 has four low-affinity MgATP binding sites [4]. However, binding of MgATP to Av_1 was not observed by us (Chapter 2).

References

1. Bulen, W.A., Burns, R.C. & LeComte, J.R. (1965) Proc.Nat.Acad.Sci. USA 53, 532-539.
2. Bui, P.T. & Mortenson, L.E. (1969) Biochemistry 8, 2462-2465.
3. Tso, M.-Y.W. & Burris, R.H. (1973) Biochim.Biophys.Acta 309, 263-270.
4. Miller, T.W., Robson, R.L., Yates, M.G. & Eady, R.R. (1980)

Can.J.Biochem. 58, 542-548.

5. Hageman, R.V., Orme-Johnson, W.H. & Burris, R.H. (1980) Biochemistry 19, 2333-2342.
6. Thorneley, R.N.F. & Lowe, D.J. (1983) Biochem.J. 215, 393-403.
7. Thorneley, R.N.F. (1975) Biochem.J. 145, 391-396.
8. Thorneley, R.N.F. & Cornish-Bowden, A. (1977) Biochem.J. 165, 255-262.
9. Lowe, D.J. & Thorneley, R.N.F. (1984) Biochem.J. 224, 877-886.
10. Eady, R.R., Lowe, D.J. & Thorneley, R.N.F. (1978) FEBS Lett. 95, 211-213.
11. Robson, R.L. (1984) FEBS Lett. 173, 394-398.
12. Van Dongen, M., personal communication.
13. Mortenson, L.E. & Thorneley, R.N.F. (1979) Annu.Rev.Biochem. 48, 387-418.
14. Smith, B.E., Thorneley, R.N.F., Eady, R.R. & Mortenson, L.E. (1976) Biochem.J. 157, 439-447.

Samenvatting

Naast water bepaalt de hoeveelheid stikstof die in de bodem aanwezig is in de meeste gevallen de produktiviteit van de landbouwgebieden op aarde. Het is van belang dat stikstof in een voor de plant bruikbare vorm beschikbaar is. Op het eerste gezicht lijkt het vreemd dat er een tekort aan stikstof kan optreden, omdat 80% van de lucht om ons heen uit stikstofgas bestaat. Maar voor nagenoeg alle vormen van leven is de stikstof zoals die in de lucht voorkomt, namelijk als het molekuul N_2, niet bruikbaar. Planten en de meeste mikroorganismen verkrijgen hun stikstof in het algemeen uit verbindingen zoals ammonia (NH_3) en nitraat (NO_3). Dieren verkrijgen de stikstof die ze nodig hebben om te kunnen groeien en funktioneren door het eten van planten en andere dieren. De reden waarom elk levend wezen stikstof nodig heeft is gelegen in het feit dat twee van de meest essentiële bestanddelen van de cel stikstof bevatten, namelijk eiwitten en nucleïnezuren (DNA, RNA). Slechts een beperkt aantal mikroorganismen is in staat om stikstofgas uit de lucht (N_2) om te zetten in ammonia. Dit proces noemt men stikstofbinding. Ammonia wordt als N-bron gebruikt voor de synthese van stikstofhoudende verbindingen. De stikstofbindende bakteriën komen in allerlei vormen voor, maar de belangrijkste voor de landbouw zijn die bakteriën die in de wortelknolletjes van vlinderbloemige planten leven in symbiose met de plant. Zonder de wetenschappelijke achtergrond te kennen maakten de Romeinen reeds gebruik van zogenaamde wisselbouw, dit wil zeggen, door het afwisselend verbouwen van vlinderbloemige gewassen als klaver en erwteplanten en niet-vlinderbloemige gewassen zoals graan, werd de vruchtbaarheid van de bodem op peil gehouden. Het duurde echter nog tot in de 19e eeuw voordat ontdekt werd dat wortelknollen bakteriën bevatten die stikstof uit de lucht om kunnen zetten in een stikstofverbinding die door de plant gebruikt kan worden.

In de huidige, intensieve landbouwgebieden wordt voor een belangrijk deel gebruik gemaakt van (stikstof) kunstmest om aan de stikstofbehoefte van de plant te kunnen voldoen. De industriële produktie van kunstmest gebeurt volgens het Haber-Bosch proces: uitgaande van de grondstoffen N_2 en H_2 wordt bij hoge druk (350-1000 atmosfeer) en een hoge temperatuur (±350°C) ammonia gevormd in aanwezigheid van een ijzer bevattende katalysator. Door de hoge energiekosten die gepaard gaan met dit proces, evenals de hoge investeringskosten die nodig zijn voor het opzetten van een kunstmestfabriek (het is alleen grootschalig ekonomisch uit te voeren) en de nood-

zaak van opslag en transport van het geproduceerde kunstmest, is de produktie van kunstmest en dus het gebruik ervan in de landbouw voor de meeste ontwikkelingslanden ekonomisch niet haalbaar. Om het toenemende aantal mensen op deze wereld van voedsel te kunnen voorzien is het echter nodig dat de landbouwproduktie toeneemt. Dit houdt in dat ook de stikstofgift aan de bodem drastisch moet stijgen. Aangezien de huidige methoden van kunstmestproduktie gebruik maken van niet onuitputtelijke bronnen van energie (fossiele brandstoffen), is het belangrijk dat onderzoek gedaan wordt met als doel de biologische stikstoffixatie te verbeteren, zodat het gebruik van kunstmest beperkt kan blijven. Biologische stikstofbinding is een proces dat gedreven wordt door een onuitputtelijke energiebron namelijk zonlicht. Bovendien wordt het biologisch gebonden stikstof efficiënt gebruikt en spoelt niet direkt uit.

Het enzym, dat in stikstofbindende bakteriën de omzetting van N_2 in NH_3 katalyseert noemt men nitrogenase. Het in dit proefschrift beschreven onderzoek heeft zich voornamelijk gericht op het werkingsmechanisme van dit enzym. Omdat de eigenschappen van nitrogenase uit de verschillende soorten stikstofbindende bakteriën nauwelijks verschillen, en omdat de bakterie Azotobacter vinelandii gemakkelijk op grote schaal gekweekt kan worden, zijn de in dit proefschrift beschreven eksperimenten uitgevoerd met nitrogenase uit deze bakterie. Tijdens de zuivering van nitrogenase wordt het enzym in twee komponenten gescheiden, vaak aangeduid met komponent 1 en komponent 2. Beide komponenten zijn nodig voor de omzetting van N_2 in NH_3. Verder zijn er voor de reduktie van N_2 tot NH_3, ATP en een sterk reduktiemiddel nodig.

In het Haber-Bosch proces wordt de energie die nodig is om het inert N_2 molekuul te reduceren geleverd door een hoge temperatuur. Daarentegen vindt de omzetting van N_2 in NH_3 door het enzym nitrogenase plaats bij kamertemperatuur. ATP is hierbij de leverancier van de benodigde energie. ATP is een in de cel voorkomende energierijke verbinding. Deze vastgelegde energie kan gebruikt worden in energie vereisende reakties. Hierbij wordt een fosfaatgroep afgesplitst en ontstaat er ADP. Voor de omzetting van één molekuul N_2 in twee molekulen NH_3 is de hydrolyse van 16 molekulen ATP nodig. De omzetting van N_2 in NH_3, en ook de hydrolyse van ATP, treedt alleen op wanneer beide komponenten van nitrogenase aanwezig zijn. De afzonderlijke komponenten kunnen geen ATP hydrolyseren.

In de hoofdstukken 2 en 3 staan de eigenschappen beschreven van de binding van ATP en ADP aan komponent 2. Komponent 1 bindt geen adenine

nukleotiden. De elektronen die nodig zijn voor de omzetting van N_2 in NH_3 worden via komponent 2 naar komponent 1 getransporteerd. Men neemt aan dat de substraat reduktie plaatsvindt op komponent 1 van nitrogenase.
De hydrolyse van ATP tijdens de nitrogenase reaktie vindt plaats wanneer een elektron wordt overgedragen van komponent 2 naar komponent 1. Omdat er geen elektronen-overdracht plaatsvindt in afwezigheid van ATP, wordt aangenomen dat de ATP hydrolyse nodig is voor deze elektronen-overdrachtsreaktie. In hoofdstuk 4 wordt aangetoond dat nitrogenase ook ATP hydrolyseert wanneer er geen elektronen-overdracht tussen beide komponenten plaatsvindt. Dit was een eerste aanwijzing dat de ATP hydrolyse mogelijk nog een andere funktie heeft behalve een direkte deelname in de elektronen-overdrachtsreaktie. Een tweede aanwijzing werd verkregen uit eksperimenten waarin aangetoond werd dat, alhoewel komponent 2 slechts twee bindingsplaatsen bezit voor ATP, er vier molekulen ATP binnen 60 millisekonden gehydrolyseerd worden door het nitrogenase kompleks. Dit suggereert dat er twee ekstra ATP bindingsplaatsen ontstaan, wanneer komponent 1 en komponent 2 een kompleks vormen. Deze ATP's zouden mogelijk rechtstreeks betrokken kunnen zijn bij de omzetting van N_2 in NH_3 op komponent 1.

In hoofdstuk 5 wordt verder ingegaan op de betrokkenheid van ATP in de nitrogenase reaktie. In het huidige model wordt aangenomen dat de langzaamste stap van de nitrogenase katalyse de snelheid is waarmee het nitrogenase kompleks verplicht moet dissociëren na de overdracht van elektronen van komponent 2 naar komponent 1. De eksperimenten beschreven in hoofdstuk 5 suggereren dat de snelheidsbepalende reaktiestap onder bepaalde kondities anders is, namelijk de dissociatiesnelheid van ADP van Av_2.

Omdat een aantal kenmerken van de ATP hydrolyse door nitrogenase terug te vinden zijn in die van een ander eiwit dat ATP hydrolyseert/synthetiseert, de zogenaamde proton ATPase, worden in hoofdstuk 6 de mogelijke overeenkomsten tussen beide eiwitten wat betreft het mechanisme van ATP hydrolyse bediskussieerd.

Curriculum vitae

Jan Cordewener werd op 26 juli 1954 te Ulestraten geboren. In 1972 behaalde hij het diploma HBS-B aan het St. Maartenscollege in Maastricht. In hetzelfde jaar werd met de studie Scheikunde begonnen aan de Katholieke Universiteit te Nijmegen. Het kandidaatsexamen (S2) werd afgelegd in 1976 en het doctoraalexamen, met als hoofdvak Biochemie (Prof.dr. J.H. Veerkamp) en als bijvakken Chemische Cytologie (Dr. A.C.M. Pieck) en Biofysische Chemie (Dr. S.H. de Bruin) in 1980.

Vanaf juli 1980 was hij vier jaren verbonden aan het Laboratorium voor Biochemie van de Landbouwuniversiteit te Wageningen als wetenschappelijk ambtenaar in dienst van Z.W.O.

In 1985 was hij een half jaar in dienst van de Landbouwuniversiteit voor het geven van onderwijs bij de vakgroep Biochemie.

Sinds december 1985 is hij werkzaam aan het Laboratorium voor Erfelijkheidsleer van de Landbouwuniversiteit, waar hij participeert in een door DSM gefinancierd onderzoeksprojekt gericht op de industriële toepassing van biokatalysatoren met mono-oxygenase aktiviteit.