Coupling of MgATP hydrolysis and electron transfer in the pre-steady-state phase of the nitrogenase reaction

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Proefschrift

ter verkrijging van de graad van doctor
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STELLINGEN

1. Geen enkel gepubliceerd rapid quench onderzoek geeft bruikbare informatie omtrent de rol van MgATP in de katalytische cyclus van nitrogenase.

   - Eady, R.R., Lowe, D.J. & Thorneley, R.N.F. (1978) *FEBS Lett.* 95, 211-213.
   - Hageman, R.V., Orme-Johnson, W.H. & Burris, R.H. (1980) *Biochemistry* 19, 2333-2342.
   - Cordewener, J., Ten Asbroek, A., Wassink, H., Eady, R.R., Haaker, H. & Veeger, C. (1987) *Eur. J. Biochem.* 162, 265-270.
   - Dit proefschrift, hoofdstuk 3.

2. De waarneming dat het menselijk lichaam in staat is nortestosteron te produceren, zet bij de dopingcontrole op dit anabool steroïd enige vraagtekens.

   - Reznik, Y., Herrou, M., Dehennin, L., Lemaire, M. & Leymarie, P. (1987) *J. Clin. Endocrinol. Metab.* 64, 1086-1088.

3. De studie van Cordewener *et al.* laat zien dat de reductant-onafhankelijke ATPase activiteit van nitrogenase te laag is om, zoals door Thorneley *et al.* is voorgesteld, als algemene verklaring te dienen voor hoge ATP/2e ratio's.

   - Thorneley, R.N.F., Ashby, G.A., Howarth, J.V., Millar, N.C. & Gutfreund, H. (1989) *Biochem. J.* 264, 657-661.
   - Cordewener, J., Krüse-Wolters, M., Wassink, H., Haaker, H. & Veeger, C. (1988) *Eur. J. Biochem.* 172, 739-745.

4. Ward en De Boer laten onbewust zien dat de door hun uitgevoerde dot-blot hybridisatie van een probe op het betreffende doelorganisme seq, en gemengd met andere organismen sterk afhankelijk is van de achtergrond; bijgevolg is de gegeven detectiegrens niet algemeen.

   - Ward, L.J. & De Boer, S.H. (1990) *Phytopathology* 80, 665-669.

5. De structuurgenerator van het CHEMICS systeem geeft in een aantal gevallen onjuiste resultaten.

   - Funatsu, K., Miyabayashi, N. & Sasaki, S. (1988) *J. Chem. Inf. Comput. Sci.* 28, 18-28.

6. Uit de studie van Wernars *et al.* waarin wordt getracht om *Listeria monocytogenes* in kaas aan te tonen m.b.v. de polymerase chain reaction (PCR) en primers voor het "delayed type hypersensitivity factor" (DTH), blijkt dat de euforie aangaande het gebruik van PCR-technieken voor detectiedoeleinden voorbarig is geweest.

   - Notermans, S., Chakraborty, T., Leimeister-Wächter, M., Durfrenne, J., Heuvelman, C.J., Jansen, W., Wernars, K. & Guinee, P. (1989) *Appl. Environ. Microbiol.* 55, 902-906.
   - Wernars, K., Heuvelman, C.J., Chakraborty, T. & Notermans, S.H.W. (1991) *J. Appl. Bacteriol.* 70, 121-126.

7. De analyse door Melese, T. *et al.* van de effecten van covalente binding van 2-nitreno-ATP op de katalytische en niet-katalytische nucleotide bindingsplaatsen van CF_1 is sterk vertekend door het gebruik van 2-azido-[ß,γ-^{32}P]ATP, omdat, ook volgens de auteurs zelf, eenmaal gebonden label in 2-nitreno-AMP wordt omgezet.

   - Melese, T., Xue, Z., Stempel, K.E. & Boyer, P.D. (1988) *J. Biol. Chem.* 263, 5833-5840.

8. Het aantal vermiste leden van de Nederlandse Vereniging voor Biochemie zal sterk afnemen wanneer een vindersloon wordt geïntroduceerd.

9. Sollicitatie-advertenties waarin vrouwen nadrukkelijk wordt gevraagd te reflecteren, zijn in zoverre beledigend daar ze suggereren dat vrouwen niet zelf het initiatief zouden nemen.

10. Er bestaat bij de hedendaagse promovendi te weinig respect voor het feit dat hun begeleiders een proefschrift hebben geschreven zonder gebruik te maken van een tekstverwerker.

Richard Mensink
Wageningen, 10 januari 1992

Voorwoord

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Tot slot wil ik mijn ouders bedanken voor hun belangstellende zorg omtrent de voortgang van het onderzoek.

CONTENTS

Abbreviations

$\Delta A(430)$	absorbance change at 430 nm accompanying reduction or oxidation of the nitrogenase proteins
AMP	adenosine 5'-monophosphate
ADP	adenosine 5'-diphosphate
ATP	adenosine 5'-triphosphate
BPS	bathophenantroline disulphonate
ΔG°	standard Gibbs energy
ΔH°	standard reaction enthalpy
ΔS°	standard reaction entropy
$\Delta G^{\circ\ddagger}$	standard Gibbs energy of activation
$\Delta H^{\circ\ddagger}$	standard enthalpy of activation
$\Delta S^{\circ\ddagger}$	standard entropy of activation
DOC	deoxycholic acid
E_m	midpoint redox-potential
EPR	electron paramagnetic resonance
FeMoco	iron-molybdenum cofactor from component I of nitrogenase
h	Planck constant
Hepes	4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid
k	rate constant
κ	transmission coefficient for electron transfer
k_B	Boltzmann constant
k_{obs}	observed rate constant
K	equilibrium constant
λ_{in}	inner-shell reorganization energy
λ_{out}	outer-shell reorganization energy
ν	frequency factor for nuclear motion
N_3	total of azido and tetrazol tautomer
P_i	inorganic phosphate
R	gas constant
SDS	sodium dodecyl sulfate
T	temperature in Kelvin
Tes	2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-amino}ethane
Tris	tris(hydroxymethyl)aminoethane

The MoFe and Fe proteins of the nitrogenase from *Azotobacter vinelandii* (Av), *Azotobacter chroococcum* (Ac), and *Klebsiella pneumoniae* (Kp) are referred to as respectively Av1 and Av2, Ac1 and Ac2, Kp1 and Kp2.

CHAPTER 1

INTRODUCTION

General

In 1888 Hellriegel and Wilfarth published their discovery of biological nitrogen fixation [1]. In the following decades it became clear that the enzyme system responsible for nitrogen fixation is labile outside the cell [2]. For this reason, researchers investigating nitrogen fixation employed intact nodule systems or bacterial cultures for their experiments. A major step forward in nitrogen fixation research has been the preparation of cell-free extracts with nitrogen fixing activity [3,4]. Further work showed that the nitrogenase complex comprises a molybdenum-iron containing protein ($\alpha_2\beta_2$) and an iron containing protein (γ_2) [5]. Recently, the presence of two more nitrogenases has been demonstrated. One is a vanadium [6] containing variety and the other contains only iron [7]. Besides being genetically distinct, these nitrogenases have different catalytic properties.

Exploratory studies with crude cell-free extracts of the nitrogenase complex established that the presence of MgATP is required for nitrogen fixing activity [8] and is hydrolysed in the catalytic process [9]. Pyruvate added to cell-free extracts was shown to be involved in the generation of MgATP and to act as the primary source of electrons [10]. *In vivo* a ferredoxin or flavodoxin mediates the electron transfer to the nitrogenase system [11]. The discovery that the non-physiological inorganic reductant dithionite can act as a source of electrons for the nitrogenase system has eased experimental work on the nitrogenase reaction [12]. Dilworth [13] discovered that acetylene is reduced to ethylene by nitrogenase. This reaction

is now generally used as a sensitive assay for the catalytic activity of nitrogen fixing systems.

The molybdenum-iron protein

The structure of the MoFe protein (component I) has been reviewed [14,15]. The MoFe protein is an $\alpha_2\beta_2$ tetramer with a molecular weight of approximately 220 kDa. Molecular weights of the α- and ß-subunit are approximately 50 kDa and 60 kDa respectively. The prevalent view is that each $\alpha\beta$-unit acts as an independent assembly. Element analysis reveals that the MoFe protein contains 24-32 iron atoms, 24-30 acid labile sulphur atoms and two molybdenum atoms. The metal atoms are divided over two types of clusters: the so-called FeMo-cofactor (FeMoco) and the P clusters. The FeMo-cofactor in dithionite- reduced MoFe protein exhibits an EPR signal with g-values of 4.3, 3.7, 2.01 [16,17].

The molybdenum-iron containing cofactor from component I of nitrogenase is extractable [18-20]. Composition [18-20] and electrochemical properties [21] depend on the used extraction method. Elemental analysis of the FeMo-cofactor gives a composition in the range of $MoFe_{6-8}S_{4-8}$. The isolated cofactor restores catalytic activity in the genetically altered proteins Av1 UW45 and Kp1 UN109 which lack the FeMo-cofactor [18]. Reconstitution experiments have also been performed with the incompletely processed FeMo-cofactor extracted from *nifV*$^-$ Kp1 and FeMoco-deficient *nifB*$^-$ Kp1 [22]. The isolated cofactor transferred the characteristics of *nifV*$^-$ Kp1 to the FeMoco-lacking *NifB*$^-$ Kp1 mutant protein. These reconstitution experiments provide strong evidence that substrate reduction takes place on the FeMo-cofactor.

Mössbauer spectroscopy has been a valuable tool in efforts to elucidate the distribution of the Fe atoms within the MoFe protein. Smith *et al.* [23] concluded in 1974 that the Fe atoms

in dithionite-reduced Kp1 reside in three different environments (M, D, and Fe^{2+}; nomenclature according to ref. [24]), each giving rise to a specific Mössbauer signal. Münck *et al.* [24] recorded a Mössbauer signal (S component) in addition to those found by Smith [23]. This signal accounted for about 5% of the total iron content. On basis of Mössbauer spectra given by reversibly thionine oxidized Av1 a model has been proposed for the grouping of the Fe atoms within the MoFe protein [25-27]. In this model the FeMo-cofactor comprises the iron atoms which give rise to the M Mössbauer component. The iron atoms which are associated with the S, D and Fe^{2+} components are thought to be grouped in four [4Fe-4S] clusters (P clusters).

The presence of two identical P clusters in each αß-unit of the MoFe protein has recently been contended. In an elegant study McLean *et al.* [28] have characterized the Mössbauer spectra of a $^{56}Fe/^{57}Fe$ hybrid of Kp1. In this hybrid the FeMo-cofactor contained only ^{56}Fe and is therefore not detected by Mössbauer spectroscopy, whereas the remaining clusters were enriched with ^{57}Fe and could be observed by Mössbauer spectroscopy. It was demonstrated that the two Fe atoms which constitute the S magnetic component are not a component of the FeMo-cofactor. Instead, it was inferred that the P-clusters occur in two inequivalent pairs. One pair is of a $D_3(Fe^{2+})_1$-type and the other of a $D_2(Fe^{2+})_1S_1$-type. The authors reported also that the Mössbauer signal of the FeMo-cofactor gives a best fit with 6Fe/Mo, though a ratio of 8Fe/Mo has also been favoured [29].

Hagen *et al.* [30] have studied a S=7/2 EPR signal from thionine oxidized nitrogenase MoFe proteins. Their data could not be interpreted by assuming the presence of four identical P clusters. The authors argue that either two P clusters form in fact one [8Fe-8S] cluster, or that the P clusters are not identical. Recently, Bolin *et al.* [31] have probed the organization of the metal clusters in the MoFe protein from

Clostridium pasteurianum by means of X-ray diffraction spectroscopy. Their data led the authors to favour a scheme in which the two P clusters in the αß-units must be considered as one 8-Fe centre. It was also found that the FeMo-cofactor and the 8-Fe centre are separated by 19 Å. See Fig. 1 for a schematic representation of the arrangement of metal clusters and Mössbauer components in the MoFe protein as outlined above.

The function of the P clusters is obscure. Intramolecular electron transfer from the FeMo-cofactor to the P clusters has

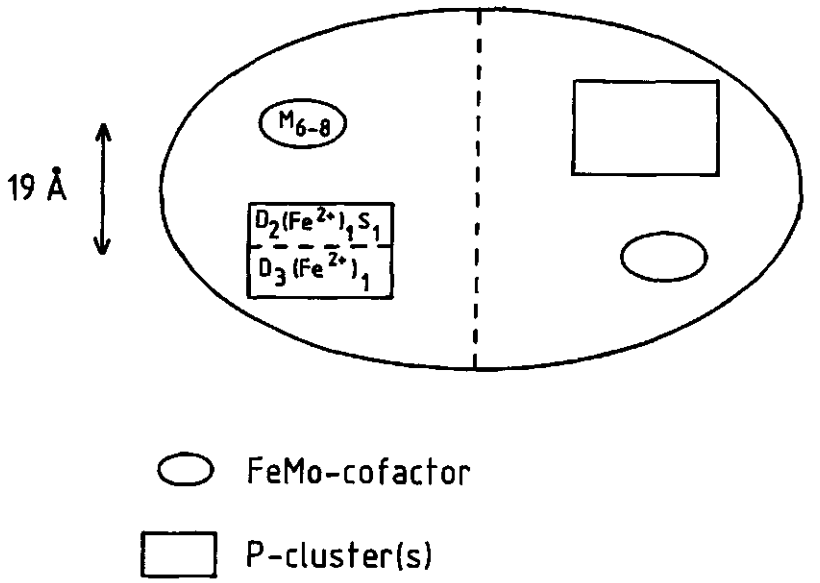

Fig. 1. *Schematic representation of the arrangement of metal clusters in the MoFe protein.* Spatial arrangement [31] of the clusters and distribution of Mössbauer components [28] have been taken from the literature. For clarity magnetic components have been omitted in one of the αß-units.

been observed in a rapid-freeze EPR study in which dithionite free Kp1 was oxidized with ferricyanide [32]. McLean *et al.* [33] propose that the P clusters may function as: a) an electron sink; b) a delocalization system for electrons; c) acceptor for electrons donated by the Fe protein; d) substrate binding site; e) a catalytic site where protons are reduced to form hydride.

No firm experimental data are available yet on which an allocation of the metal clusters over the subunits of the MoFe protein can be based. It has been inferred from the spectroscopic and catalytic properties of single amino acid altered Av1 [34] and Kp1 [35] that the α-subunit is associated with the FeMo-cofactor.

The Fe protein

The Fe protein (component II) is a homodimer with a molecular mass of approximately 60 kDa [14,15]. Usually the Fe protein is reported to contain one [4Fe-4S] cluster, though Braaksma *et al.* [36] have described purified Av2 with a higher iron and sulphur content. The Fe protein functions in the catalytic cycle of nitrogenase as the reductant of the MoFe protein.

Relevant biophysical properties of the Fe protein are largely determined by the iron-sulphur cluster. The dithionite-reduced [4Fe-4S] cluster is responsible for a S=1/2 EPR signal characterized by a set of three g-values around 2.0 [16,17,37]. A long standing enigma has been the intensity of the S=1/2 EPR signal. Quantization of the S=1/2 EPR signal yields a spin intensity of less than one mol electrons·mol^{-1} Fe protein [38]. This problem was solved by the observation of a S=3/2 EPR signal which had long escaped detection [39-41]. The summed spin intensities of both EPR signals is approximately equal to one mol electrons·mol^{-1} Fe protein. The intensity of the spin states responds to the presence of MgADP, MgATP, and denaturing

reagents [42]. Both spin states were found to have identical redox properties [43]. On basis of a ^{1}H NMR study Meyer [44] has concluded that the S=3/2 EPR signal is an artefact, caused by freezing the protein solution.

Hausinger & Howard [45] have selectively labelled 14 cysteinyl residues in Av2 with iodo[2-^{14}C]acetic acid. On basis of the obtained results they concluded that the [4Fe-4S] cluster in Av2 is sandwiched between the two subunits of the Fe protein and ligated to cysteines 97 and 132. The localization of amino acids ligated to the [4Fe-4S] cluster was continued in a site-specific mutagenesis study [46]. This study confirmed that cysteines 97 and 132 in Av2 are essential for catalytic activity. See Fig. 2 for a schematic representation of the structure of the Fe protein. The ligation of the [4Fe-4S]

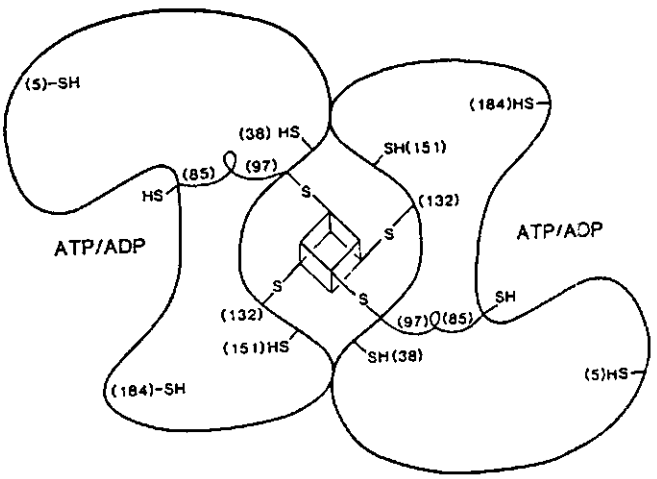

Fig. 2. *Schematic representation of the structure of the Fe protein.* From ref. [45].

cluster as given in Fig. 2 has recently been confirmed by a crystallographic structure determination [47].

A stopped-flow spectrophotometry study in which Kp2(ox)$(MgADP)_2$ was reduced with dithionite indicates that the complex between Kp2(ox) and MgADP can exist in two conformations [48]. The kinetics of MgATP induced chelation of Fe from reduced Av2 has also been explained with the assumption that free Av2 is present in two conformational states [49].

The [4Fe-4S] cluster can be converted into a [2Fe-2S] cluster by means of MgATP and the iron chelating reagent α,α'-bipyridyl [50]. Catalytic activity of the Fe protein is lost in this process.

Binding of MgADP and MgATP by nitrogenase proteins

A crucial step in understanding the mechanism by which nitrogenase functions, has been the realization that the presence of MgATP is required in the nitrogenase reaction [8]. It was later discovered that MgADP inhibits the activity of the nitrogenase complex [51,52]. As a consequence, binding of nucleotides to the nitrogenase proteins became an area of interest.

A gel equilibration study showed that the MoFe protein from *K. pneumoniae* has four binding sites for MgATP [53]. A proton NMR relaxation technique has been used to study the binding of Mn^{2+} to Kp1 [54]. Three binding sites for the manganese ion could be observed directly. Binding of Mg^{2+} could be inferred from the observation that this ion suppresses the relaxation enhancement caused by Mn^{2+}. The number of binding sites was proportional to the specific activity of the protein used. On basis of an extrapolation to the highest specific activity reported for Kp1, it was concluded that the MoFe protein has four binding sites for Mg^{2+}. This was taken as evidence that Kp1 has four binding sites for MgATP. It was

proposed that in the complex the nucleotide bridges both proteins. Miller & Eady [55] demonstrated by the column equilibration technique that dye-oxidized and dithionite-reduced Ac1 has two binding sites for MgADP. In contrast, Cordewener *et al.* [56] failed to detect binding of MgADP/MgATP to the MoFe protein from *A. vinelandii* by means of the flow dialysis technique. It has been suggested that this negative result must be attributed to a failure of the flow dialysis technique in detecting slowly dissociating nucleotides [55].

Cordewener *et al.* [56] have used the flow dialysis technique to investigate the binding of MgADP and MgATP to dithionite-reduced and dye-oxidized Av2. It was concluded that Av2, reduced as well as dye-oxidized, has two nucleotide binding sites. Both sites can be occupied by either MgADP or MgATP. These observations are in concord with the current prevailing view regarding nucleotide binding by the Fe protein of the nitrogenase complex.

It is firmly established that both MgADP and MgATP alter the conformation of the Fe protein. The conformation change is reflected in an increased sensitivity for oxygen [57], the number of reactive thiol groups [58], a change in the circular dichroism spectrum of the oxidized Fe protein [59,60], the reactivity of the [4Fe-4S] cluster with chelating agents [61], and a decrease in the midpoint potential [37]. The intensity [39] and shape [62] of the EPR signals of the Fe protein are affected by the presence of MgATP or MgADP.

No detailed information is available yet on the localization of the nucleotide binding sites on the Fe protein. A labelling study with iodo[2-^{14}C]acetic acid showed that MgATP protects cysteine 85 in Av2, which is an indication that this particular amino acid residue is located within the nucleotide binding region of the Fe protein [46], see also Fig. 2. Though binding of MgATP affects the conformation around the [4Fe-4S] cluster [61], pulsed EPR experiments show that MgATP is not in

close contact with the [4Fe-4S] cluster of Av2 [63].

Robson [64] has compared the amino acid sequence of a some nucleotide-binding proteins and a number of nitrogenase proteins. For the Fe protein it was deduced that the N-terminus, and the region centred around residues 70 and 97 are possible adenine nucleotide binding sites.

Recently, tight binding of MgADP and MgATP to the nitrogenase proteins have been investigated by the gel-filtration technique. Dithionite-reduced as well as dye-oxidized Av2 was found to bind tightly either one MgATP/mol protein or one MgADP/mol protein in gel-centrifugation experiments [65]. Miller & Eady [55] found that one mol MgADP/mol Ac1(ox) remained tightly bound during in gel-filtration experiments. It is not clear yet whether this tight binding behaviour of the nitrogenase proteins is of relevance during catalytic turnover.

Role of MgATP in the nitrogenase reaction

Elucidation of the MgADP/MgATP binding properties of the nitrogenase proteins has not been paralleled by a deeper insight in the mechanism of MgATP hydrolysis by the nitrogenase complex. EPR [16,17,66], Mössbauer [23], and stopped flow [67-70] experiments show that the presence of MgATP is obligatory for electron transfer from the Fe protein to the MoFe protein, but its mode of action is still vague.

The ratio MgATP molecules hydrolysed:pair of electrons transferred to substrate (commonly denoted as the ATP:2e ratio) has been thoroughly investigated. It is now generally accepted that the ATP:2e ratio can not fall below 4 [71]. However, the ATP:2e ratio is sensitive to the pH [71], reaction temperature [72-74], the ratio MoFe protein:Fe protein [75], and redox potential of the reaction mixture [76]. Also, ATP:2e ratios in assays with the alternative substrates cyanide [77] and methyl isocyanide [78] can be as high as 20. Notably high ATP:2e ratios

have also been reported with cross reactions between Kp1 and Cp2 [79]. Rennie *et al.* [80] discovered that antibodies against the MoFe protein inhibited both substrate reduction and the hydrolysis of ATP by the nitrogenase complex from *K. pneumoniae*. Increased ATP:2e ratios are generally explained by assuming that electron transfer and the hydrolysis of MgATP are uncoupled.

Pre-steady-state studies with the aid of the rapid quench and stopped-flow technique indicate that electron transfer from the Fe protein to the MoFe protein is accompanied by hydrolysis of MgATP [65,70,81]. Unfortunately, no consensus has been reached on the value for the ratio mol MgATP hydrolysed·mol^{-1} electrons transferred to the Fe protein. It has been demonstrated that hydrolysis of MgATP by nitrogenase occurs is a one-step process [82] and takes place at the P_{γ}-OP_{β} bond [83].

Cordewener *et al.* [84] reported that the reductant-independent ATPase activity of nitrogenase from *A. vinelandii* increases if the ratio Av2(ox) to Av1(ox) is raised. The maximal turnover rate of Av1(ox) in the ATPase reaction of dye-oxidized nitrogenase was reported to be approximately the same as that of Av1(red). It was suggested that in the reductant-independent ATPase reaction of nitrogenase the Fe protein dissociates from the MoFe protein in an inactive form. Regeneration of active Av2, possibly by displacement of MgADP by MgATP, would be the rate limiting step in the catalytic cycle of the Fe protein. By studying the kinetics of phosphate-water ^{18}O-exchange Thorneley *et al.* [85] have been able to demonstrate that reductant-independent MgATP cleavage by the nitrogenase complex is a reversible process.

Kinetic model for the nitrogenase cycle

Compared with many enzyme catalysed reactions the kinetics of the nitrogenase reaction is unusually complicated. This arises from the fact that nitrogenase comprises two proteins,

which have a complicated interaction before the reduction of dinitrogen has been accomplished. The nitrogenase reaction is characterized by some marked features which have been summarized below.

Early investigations showed that H_2 inhibits nitrogen fixation [86]. It became also clear that the reduction of dinitrogen is invariably accompanied by formation of molecular hydrogen [87]. The ratio of H_2 evolved to N_2 fixed can not fall below 1 [88], which suggests that H_2 evolution is dictated by the reaction mechanism. At low MgATP [73,89] or dithionite concentrations [89] protons are a more efficient electron acceptor than either dinitrogen or acetylene. The same effect is observed if the ratio MoFe protein to Fe protein is suboptimal [90]. These reaction conditions have in common that they decrease the rate by which the MoFe protein accumulates electrons.

Besides dinitrogen and protons, nitrogenase reduces a number of small molecules such as acetylene, CN^-, N_3^-, and cyclopropene [91]. The electron flux through nitrogenase is not influenced by the substrate.

The activity of the Fe protein in the acetylene assay is inhibited by an excess of the MoFe protein, whereas the activity of the MoFe protein is unaffected by an excess of the Fe protein [92]. Thorneley *et al.* [93] have examined the rate of acetylene reduction by a reaction mixture which contained Kp1 and Kp2 in a 1:1 ratio at various protein concentrations. The rate acetylene reduction per milligram protein drops at low protein concentrations (dilution effect). This effect must be attributed to the dissociation of the complex between the component proteins of nitrogenase. Hageman & Burris [94] have investigated the concentration dependence of the kinetics of H_2 evolution, C_2H_2 reduction, and dithionite consumption as a function of the nitrogenase concentration. The observed kinetics could be explained by assuming that both a 1:1 and 1:2 complex (MoFe

protein:Fe protein) are catalytically active and dissociate during turnover.

In the absence of reducible substrate, nitrogenase functions as a hydrogenase provided MgATP and a suitable electron donor are present [95]. Hageman & Burris [96] found that at a low Av2:Av1 ratio hydrogen evolution displays a lag period. This observation indicates that the Fe protein is a one electron donor and dissociates from the MoFe protein after intermolecular electron transfer.

Pioneering studies had shown that nitrogenase catalyses in the presence of dinitrogen an exchange reaction between molecular deuterium (D_2) and molecular hydrogen in which HD is formed [87]. The proposed reaction mechanism for this phenomenon features a reaction step in which D_2 displaces H_2 from the catalytic centre [97,98]. Displacement of N_2 by H_2 or D_2 has by Guth & Burris [99] been proposed in order to account for the observed competitive inhibition of dinitrogen reduction by molecular hydrogen.

The general view is that reduction of dinitrogen proceeds with the formation of several intermediates. None of the

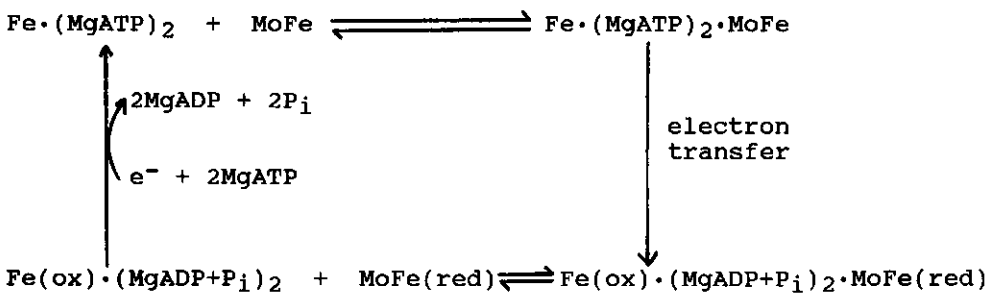

Fig. 3. *MgATP dependent reduction of the MoFe protein by the Fe protein in the Lowe-Thorneley model for the catalytic cycle of nitrogenase*. Adapted from ref. 101. Fe and MoFe denote the Fe protein and αß-unit of the MoFe protein, respectively.

intermediates have been positively identified. However, a reaction intermediate which forms hydrazine (N_2H_4) upon quenching with acid or base has been detected [100].

A kinetic model which addresses the features which have been discussed in the preceding paragraphs has been advanced by Lowe and Thorneley. It should be noted here that this model is based on kinetic data obtained with the nitrogenase from *K. pneumoniae*, some species differences in the separate rate constants can not be excluded. Lowe & Thorneley [101] set out to develop a model which describes the electron transfer from the Fe protein to the MoFe protein, see Figure 3. This model depicts the complete turnover of the Fe protein and is usually designated the "Fe protein cycle". Essential in this reaction scheme is that after electron transfer dissociation of the enzyme complex (6.4 s^{-1} at 23 °C) is rate limiting in the catalytic cycle [102].

When Lowe & Thorneley presented their model no detailed information was available yet on the release of MgADP by the oxidized Fe protein. Ashby & Thorneley [48] later demonstrated that the Fe protein is reduced before MgATP displaces MgADP.

Depending on the substrate, the MoFe protein requires from two (proton reduction) to eight (nitrogen reduction) reduction steps by the Fe protein. Lowe & Thorneley present their model for the MoFe protein cycle in three successive papers [102-104]. In a fourth paper they demonstrate that the developed model can successfully simulate the dilution effect and the influence of protein ratios on the rate of hydrogen evolution [105]. See Figure 4 for a representation of the MoFe protein cycle.

The reaction scheme depicts a stepwise reduction of substrate which is complexed to the MoFe protein ("E" in Fig. 4). The subscript 'n' denotes the number of electrons which have been directed to the catalytically active αß-unit of the MoFe protein. Each electron transfer is followed by a protonation step. Crucial in the reaction scheme is that

dinitrogen can only bind to E_3H_3 and E_4H_4 with the concomitant release of H_2. This feature explains why reduction of dinitrogen

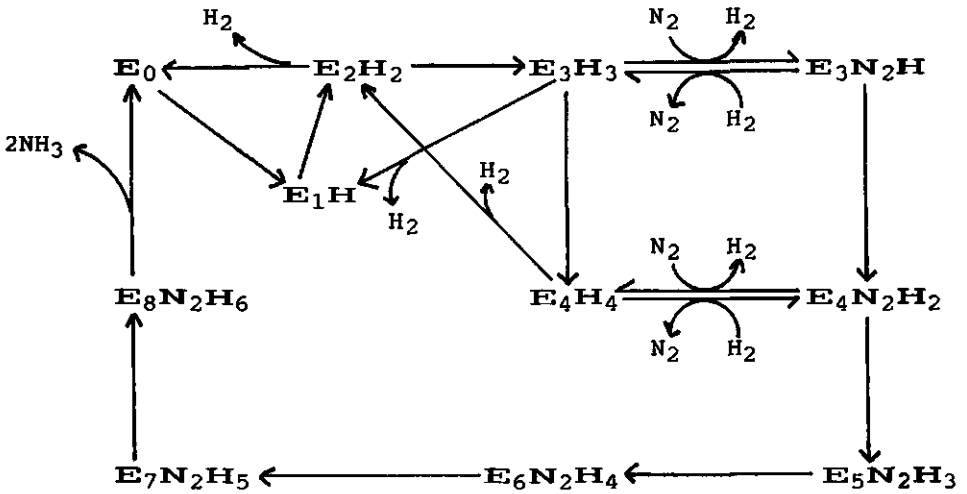

Fig. 4. *Reduction of dinitrogen by the MoFe protein according to the Lowe-Thorneley model.* Adapted from ref. 102.

is necessarily accompanied by the evolution of molecular hydrogen. Hydrogen can only be released if the MoFe protein is not associated with the Fe protein. In order to simulate their kinetic data, Lowe & Thorneley had to postulate that the Fe protein is present in two distinct forms [102-105]. Only 50% of the Fe protein present would be active in intermolecular electron transfer. It was proposed that the function of the redox-inactive form of the Fe protein is to complex the E_3H_3 and E_4H_4 species, so that the amount of H_2 released by the MoFe protein without binding dinitrogen is minimized. It is not yet known which modification renders the Fe protein redox inactive.

The model for dinitrogen reduction may not be generally

applicable to the alternative substrates of nitrogenase. Extrapolation to an infinite substrate concentration indicated that under steady-state conditions the reduction of C_2H_2 [106] and cyanide [107] are not coupled to H_2 evolution. Recently, Lowe *et al.* [108] have been able to prove by pre-steady-state experiments that C_2H_2 binds to nitrogenase without displacing H_2. However, Liang & Burris [109] reported that the reduction of azide and cyanide is accompanied by a burst of respectively 1 and 2 mol $H_2 \cdot mol^{-1}$ MoFe protein after which the production of H_2 ceases. It was proposed that these observations are associated with an activation process of the MoFe protein.

The mechanism by which MgATP facilitates the electron transfer from the Fe protein to the MoFe protein function of MgATP is still unknown and poses a challenge. From a bioenergetic point of view the answer must be sought in the elucidation of the mechanism by which the free energy accompanying the hydrolysis of MgATP is utilized to drive the electron transfer. In relation to this problem two communications are of special interest. Firstly, Cordewener *et al.* [65] have reported that dye-oxidized nitrogenase has a pre-steady-state ATPase activity identical to reduced nitrogenase. Secondly, from results obtained in a combined stopped-flow calorimetry and stopped-flow spectrometry study Thorneley *et al.* [110] concluded that at 6 °C the hydrolysis of MgATP by nitrogenase precedes electron transfer. In line with these results is a kinetic model in which the energy released by the hydrolysis of MgATP is transduced within the nitrogenase complex and induces a modification at molecular level which promotes reduction of the MoFe protein.

Affinity labelling of enzymes

The mode of substrate binding by an enzyme can be complex. Binding can be cooperative, or influenced by other molecules.

Substrate binding is usually monitored in an enzyme solution to which varying amounts of substrate are added. This approach allows the investigator to extract information about the number of binding sites involved and their affinity for substrate and inhibitors. A disadvantage is that this type of experiments provide, as a rule, no information on the localization of binding sites. Extraction of such information requires a stable enzyme-substrate complex which can be subjected to suitable analysis.

Localization of binding sites may be attempted by modifying specific amino acids [111,112]. For example, iodoacetamide reacts with the sulphydryl group of cysteinyl residues in a protein. If this results in the loss of affinity for a substrate, it can be concluded that the labelled SH-groups form a part of the substrate binding site. Probes used in this type of studies are selected on basis of their affinity for amino acids. They are therefore called group-directed reagents [112]. A refined approach is to attach the affinity group to the substrate. The substrate serves as a carrier for the affinity group and directs it to the binding site. The construct of affinity group and substrate thus acts as an active site-directed reagent [113]. Analysis of the reaction product gives information to which amino acids(s) and subunit(s) the substrate has been linked.

Affinity labels are generally very reactive and may lead to non-specific labelling of the enzyme. On the other hand, the substrate binding site may possess no amino acid residues which reacts with the affinity group of the label. Ideally, the affinity group should react with a wide range of groups. This demand is met by photogenerated nitrene radicals ($N^{\cdot}$) [114]. Nitrene radicals are generated by irradiation of azido ($R-N=N^{+}=N^{-}$) compounds with ultraviolet light. These nitrene radicals react also with water, so free radicals are trapped before they label the protein aspecifically.

Azido analogues of AMP, ADP, and ATP are often used in studies on nucleotide binding sites on enzymes. Investigators can resort to several azido-analogues of these nucleotides [113]. The derivatives with the azido group directly placed in the 2, or 8 position of the adenine moiety are often chosen. The

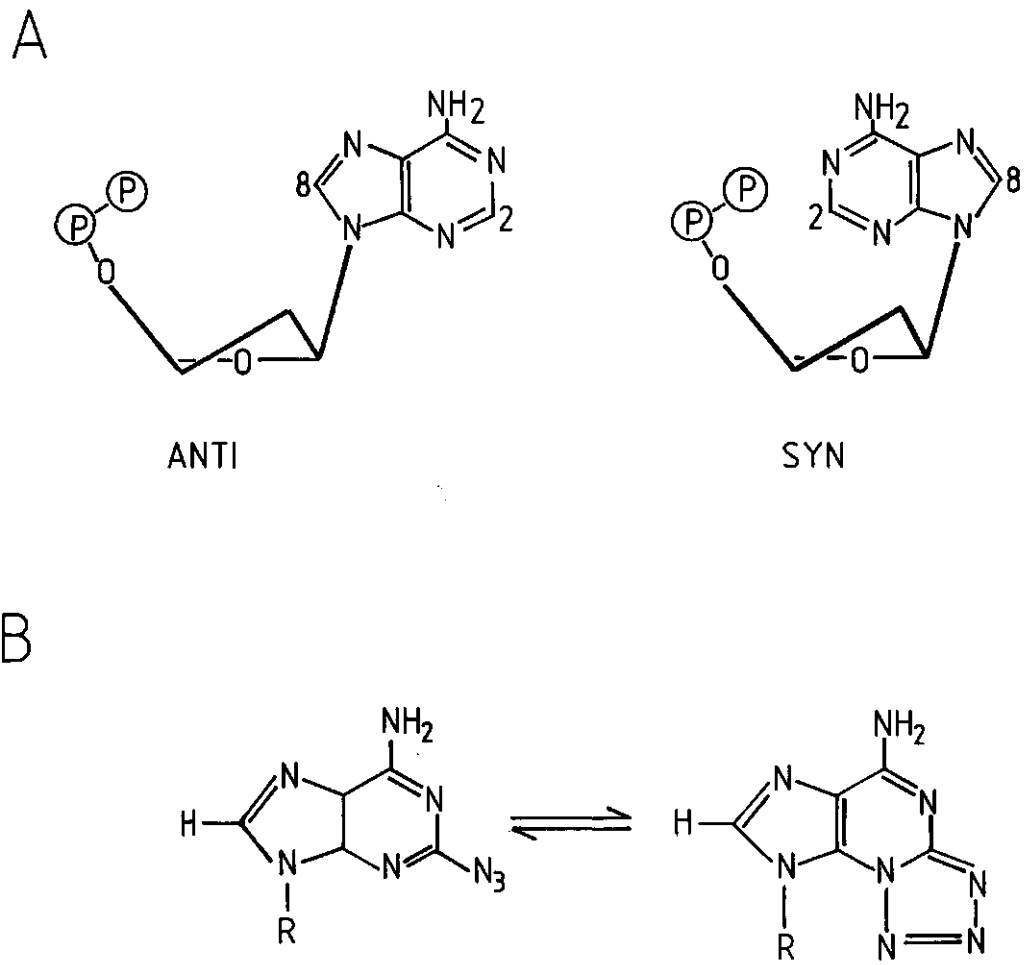

Fig. 5. *(A) The syn and anti conformation of ATP; (B) The azido/tetrazolo equilibrium of 2-azido-ATP.*

8-azido analogues came first into use [115]. Though 8-azido nucleotides have been utilized with success [e.g. 115-118], their use has been limited because 8-substituted adenosine phosphates are poor substrates as compared with the unmodified nucleotides. This can be related to the conformation of 8-azido-nucleotides. In solution ADP and ATP exist in a dynamic equilibrium between the *syn* and *anti* conformation (Fig. 5A) with a preference for the *anti* conformation. Substitution of an azido group at the 8-position shifts the equilibrium toward a static *syn* conformation. The conformational change may be accompanied by an decreased affinity for the nucleotide binding site. Substitution at the 2-position results in an analogue with a preference for the *anti* conformation [113]. However, the azido group exists in equilibrium with the photo inactive tetrazolo tautomer, see Fig. 5B. This equilibrium is influenced by the pH and solvent composition [119].

Outline of this thesis

The kinetics of electron transfer between metalloproteins is now a well established field of interest for theoretical and experimental chemists. Before metalloproteins can exchange an electron the reactants have to form a complex in a bimolecular reaction. Once the complex is formed, the rate of electron transfer is largely determined by the separation of the redox centres and the free energy change for electron transfer [120].

The nitrogenase complex represents a unique electron transferring system, since it is the only known example in which electron transfer between two metalloproteins requires the presence of MgATP. A number of experimental studies on the role of MgATP in the catalytic cycle of nitrogenase have been published in the past [65,70,81,110]. However, none of these studies have provided firm experimental data which can used to developed a model for the mechanism by which the free energy of

MgATP hydrolysis is used to drive electron transfer within the nitrogenase complex. The experiments presented in this thesis were performed with the aim to obtain more information on the pre-steady-state ATPase activity of nitrogenase complex in relation to the pre-steady-state electron transfer.

Chapter 2 deals with a pioneering study on the use of 2-azido analogues as photoaffinity label for the nucleotide binding sites on the nitrogenase complex. The major aim of these investigations has been to detect nucleotide binding by the MoFe protein of the nitrogenase complex.

In Chapter 3 a reinvestigation of the pre-steady-state ATPase activity of the reduced and dye-oxidized nitrogenase complex is presented. It has been found that the data on the pre-steady-state ATPase activity of the nitrogenase from *A. vinelandii* reported by Cordewener *et al.* [65] have been partly in error due to an apparatus artefact. It is also demonstrated that pre-steady-state proton production by the nitrogenase complex can be monitored by monitoring the absorbance change of the pH indicator cresol red in a stopped-flow spectrophotometer.

In Chapter 4 the temperature dependence of the extent and rate of MgATP dependent electron transfer within the nitrogenase complex have been analyzed. It is demonstrated that the progression curve of the oxidation of the Fe protein from *A. vinelandii* is mono-exponential in the temperature range between 6.0 °C and 30.7 °C. This observation invalidates any kinetic model in which the hydrolysis of MgATP and electron transfer are represented in a consecutive reaction mechanism characterized by rate constants of the same order of magnitude. The data presented Chapter 4 have been analyzed with the assumption that electron transfer from Av2 to Av1 is reversible. An interpretation within the framework of the transition-state-theory reveals that electron transfer from the Fe protein to the MoFe protein is characterized by a high activation entropy.

The effect of high NaCl concentrations on the MgATP

induced electron transfer from Av2 to Av1 is summarized in Chapter 5. It was found that NaCl (100-500 mM) suppresses the absorbance change due to electron transfer from Av2 to Av1, thus suggesting partial reduction of the FeMo-cofactor. However, it was demonstrated that the extent of reduction of FeMoco as observed by the rapid-freeze EPR technique is significantly larger than suggested by stopped-flow spectrophotometry. These observations will be discussed with reference to the current model for electron transfer within the nitrogenase complex.

A general discussion of the results presented in this thesis is given in Chapter 6, followed by a summary in Dutch.

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CHAPTER 2

AFFINITY LABELLING OF DYE-OXIDIZED NITROGENASE PROTEINS BY 2-AZIDO ADENOSINE PHOSPHATES

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SUMMARY

Dye-oxidized nitrogenase from *Azotobacter vinelandii* hydrolyses 2-azido-MgATP as efficiently as MgATP. The affinity of the dye-oxidized Fe protein from *Azotobacter vinelandii* for the 2-azido-[α-^{32}P] analogues of MgAMP, MgADP, and MgATP has been investigated. It is demonstrated by autoradiography that the nucleotide binding sites on the dye-oxidized Fe protein are labelled by the 2-azido analogues of MgADP and MgATP. It is also shown that the 2-azido group has an affinity for the [4Fe-4S] cluster in the Fe protein. Probably a bond is formed between the azido group and cystein residues normally ligated to the iron-sulphur cluster. The label bound to this site is removed by 2-mercaptoethanol, presumably by reduction of the S-N bond formed between the 2-azido nucleotide and the labelled cysteine residue.

Labelling experiments with the 2-azido analogues of MgAMP, MgADP, and MgATP provided no evidence for the presence of nucleotide binding sites on the MoFe proteins from *Azotobacter vinelandii* and *Azotobacter chroococcum*.

INTRODUCTION

Nitrogenase is the enzyme system responsible for the biological reduction of dinitrogen to ammonia. Two oxygen sensitive metalloproteins constitute the complete enzyme system. The larger protein (MoFe protein) is a tetrameric ($\alpha_2\beta_2$, M_r ±220 kDa) unity. It contains per αß-unit one FeMo-cofactor which is thought to be the active site for substrate reduction. Preparations of the smaller dimeric (γ_2, M_r ±60 kDa) iron-sulphur protein usually contains one [4Fe-4S] cluster, though also higher Fe-contents have been reported [1]. The Fe protein functions as reductant for the MoFe protein. For catalytic activity the presence of both nitrogenase proteins, MgATP, and a strong reductant are required. Comprehensive reviews of the catalytic mechanism [2] and relevant properties of the nitrogenase proteins [3] have been published.

It is firmly established that the Fe protein has two binding sites which can be occupied by either MgADP or MgATP [4]. Nucleotide binding by the MoFe protein is less well documented. Binding sites for MgADP have been found on the MoFe protein from *Klebsiella pneumoniae* [5,6] and *Azotobacter chroococcum* [7]. However, no nucleotide binding sites could be observed on the MoFe protein from *Azotobacter vinelandii* [4]. On basis of amino acid sequence analysis Robson [8] has predicted a nucleotide binding site on the ß-subunit of the MoFe protein. It has recently been found that Av2 in the reduced and dye-oxidized form has the capacity to bind tightly either one MgADP or one MgATP molecule [9]. Miller & Eady [7] demonstrated tight binding of MgADP by the dye-oxidized MoFe protein from *A. chroococcum*. It has been suggested that MgATP binds in a bridging mode to the nitrogenase complex [6,7].

Photoaffinity labelling [10] with the aid of the 2-azido analogues of MgADP and MgATP is a yet unexplored, though potentially powerful technique to locate the nucleotide binding

sites on the nitrogenase proteins. The major advantage of the 2-azido affinity labels is that the *anti* conformation of the nucleotide is retained [11]. However, their use is complicated by the fact that the 2-azido group isomerizes to the 2-tetrazolo group, which is not photoreactive and will compete with the azido tautomer [11]. In this paper we report the results of an investigation on the affinity of dye-oxidized nitrogenase proteins for the 2-N_3-[α-^{32}P] analogues of MgAMP, MgADP, and MgATP.

MATERIALS AND METHODS

Synthesis of 2-N_3-[α-^{32}P] nucleotide analogues

The synthesis of 2-N_3-[α-^{32}P]AMP was performed essentially by a published procedure [12]. Pyrophosphate was coupled to 2-N_3-[α-^{32}P]AMP according to the method of Hoard & Ott [13] to give 2-N_3-[α-^{32}P]ATP with about 60% yield. All glassware was siliconised before use in order to prevent absorption of ^{32}P. 2-N_3-[α-^{32}P]ADP was obtained by dephosphorylation of 2-N_3-[α-^{32}P]ATP in a hexokinase-glucose system. The affinity labels were analyzed spectrophotometrically [11] and enzymatically [14] and were found to be at least 95% pure. Preparations were stored at 20 °C as a water solution of the triethylammonium salt (pH 7.0).

Analytical methods

Nitrogenase proteins were isolated from *Azotobacter vinelandii* ATCC 478 and oxidized as described elsewhere [4]. Concentrations of the isolated component proteins were estimated by the microbiuret method [15] after a precipitation step with deoxycholic acid and trichloroacetic acid [16]. Bovine serum

albumin was used as a standard. Nitrogenase activities were measured at 30 °C as described by Braaksma *et al.* [17]. Dye-oxidized Av1 and Av2 preparations had specific activities of 2500 and 1800 nmol ethylene produced·min^{-1}·mg^{-1} protein respectively.

The microbiuret requires an impracticable amount of sample at protein concentrations less than 5 mg/ml. For this reason the more sensitive Bradford assay [18], with bovine serum albumin as standard, has been used to estimate the protein concentration in samples of photolabelled nitrogenase proteins. A comparison between the microbiuret and Bradford assay established that the latter method underestimates the protein concentration in Av2 preparations by 20%. Protein concentrations in Av2 samples obtained with the Bradford method have been corrected accordingly.

Reductant-dependent and reductant-independent ATPase activities have been measured enzymatically at 23 °C as described elsewhere [19].

The effect of labelling on the stability of the [4Fe-4S] clusters of the Fe protein has been investigated by means of the reaction between Fe^{2+} and bathophenanthrolinedisulfonate (BPS) [20] in a total reaction volume of 1100 µl. Extruded Fe^{3+} was converted to Fe^{2+} by the addition of dithionite (final conc. 4.5 mM). BPS was added to 0.9 mM and the absorbance change was recorded at 535 nm. Subsequently, iron still ligated to the protein was released by the addition of SDS to 0.09% (w/v), after which the accompanying absorbance change was monitored. The absorption coefficient used for the Fe^{2+}-BPS complex was ϵ_{535}=22.14 mM^{-1}·cm^{-1}.

Polyacrylamide gel electrophoresis was carried out according to Laemmli [21]. Protein samples were dissolved at 90 °C in 250-750 µl 50 mM Tes/NaOH pH 7.4, 4% (w/v) SDS, 1 mM EDTA, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 0.001% (w/v) bromophenol blue. For optimal resolution 10-20 µg protein

was run through a separating slab gel of 15% acrylamide with 0.09% bisacrylamide. The separating gel was topped by a 5% acrylamide stacking gel. After staining with Coomassie Brilliant Blue, the slab gels were dried and used to prepare an autoradiograph. In some instances the amount of label in the protein bands in the slab gel was determined. For this purpose the stained protein bands were cut out and transferred into 1 ml 90 % Protosol (NEN, DuPont) and solubilized overnight at 37 °C. After neutralizing the Protosol with 100 µl glacial acetic acid the amount of radioactivity in the extract was counted.

Photoaffinity labelling of nitrogenase proteins

In all experiments dye-oxidized nitrogenase proteins were used since the azido-group is reduced by dithionite. The affinity label was diluted to the required concentration in argon saturated 50 mM Tes/NaOH containing 5 mM $MgCl_2$ (final pH 7.4) under an argon atmosphere in a quartz cuvette capped with a Subaseal rubber closure. In competition experiments the unmodified nucleotide was also added at this stage. Non-radioactive 2-azido-analogues have been used to investigate the effect of labelling on the enzymatic activities of the nitrogenase proteins. After flushing the cuvette with argon for an additional 5 minutes, the nitrogenase protein was added to a concentration as given in the legends to the tables and figures. The reaction mixture was positioned 2 cm in front of a CAMAG TL-900/U ultraviolet lamp and irradiated at 254 nm for 4 minutes. If required, the protein labelled by the 2-azido-[α-^{32}P] nucleotide was precipitated in an Eppendorf reaction vessel with trichloroacetic acid (final concentration 7-15%:w/v). After centrifugation the pellet was resuspended in 500 µl trichloroacetic acid 5% (w/v) and centrifuged. Finally the protein pellet was washed with 500 µl H_2O, centrifuged, and dissolved in sampling buffer. Covalently bound label was

quantified by counting radioactivity in samples of 25 µl or 50 µl.

Gel centrifugation experiments

The amount of label bound to Av2 prior to acid precipitation and electrophoresis was determined by separating unbound label from labelled protein by the gel centrifugation technique of Penefsky [22]. After irradiation of 300 µl protein solution in the presence of the required 2-N_3-nucleotide, the sample was transferred into an Eppendorf vessel and SDS was added to a final concentration of 0.1% (w/v). To guarantee the release of any tightly bound 2-N_3-nucleotide, MgADP was added to 5 mM. Subsequently, the protein solution was transferred to a 5 ml syringe packed with Sephadex G-50 (coarse) equilibrated with 50 mM Tes/NaOH, 200 mM NaCl, 0.1% SDS, final pH 7.4. The tip of the column was placed in an Eppendorf vessel which was allowed to stand in a test tube. The column was centrifuged for 60 seconds (1000xg). SDS and MgADP were added in the same quantities as had been done prior to running the gel centrifugation. After the sample had been incubated at 35 °C for a period of 90 minutes the solution was run over a second Penefsky column. Subsequently, the protein concentration and radioactivity were determined. The stability of the covalent bond between the protein and label in the presence of 2-mercaptoethanol has been investigated in parallel trials in which 2-mercaptoethanol was added to 5% (v/v) after each centrifugation step.

Detection of slowly dissociating 2-N_3-MgADP

A Bio-Gel-6PG (Bio Rad) column (8x1 cm) was equilibrated with argon saturated 50 mM Tes/NaOH, 5 mM $MgCl_2$, pH 7.4. The buffer contained 50 mM NaCl and 200 mM NaCl in experiments with

Ac1 and Av1 respectively. No NaCl was added in experiments with Av2. Nitrogenase proteins were equilibrated for 3 minutes with 2-N_3-[α-^{32}P]MgADP. In competition experiments MgADP was added simultaneously with 2-N_3-[α-^{32}P]MgADP. Protein and nucleotide concentrations are given in Table 3. The mixture (120-220 μl) was loaded on the column and eluted with argon saturated buffer; 0.4 to 1.0 ml fractions were collected. In each fraction the protein concentration was estimated by the Bradford assay. The amount of label coeluted with the protein was measured by counting radioactivity.

In some instances the labelling efficiency by tightly bound nucleotide was determined. For this purpose the effluent containing the main fraction of protein with bound 2-N_3-[α-^{32}P]MgADP was irradiated with ultraviolet light immediately after collecting. Subsequently a sample was taken for counting the radioactivity. To remove free label, the remaining protein was precipitated with trichloroacetic acid and washed. The protein pellet was subsequently dissolved at 90 °C in sampling buffer and radioactivity was counted.

Materials

All chemicals were of the purest grade available and were obtained from commercial sources. 2-Chloroadenosine was from Sigma. $H_3[^{32}P]O_4$ was supplied by Dupont-NEN. The Bradford reagent was supplied by Biorad. Lactate dehydrogenase, pyruvate kinase and glucose oxidase (grade III) were purchased from Boehringer.

RESULTS

Reductant-independent hydrolysis of 2-azido-MgATP

The reductant-independent ATPase activity of the nitrogenase from *A. vinelandii* has been used to check whether 2-azido-MgATP is a substrate for the nitrogenase complex. In Fig. 1 the hydrolysis rates of MgATP and 2-azido-MgATP are compared. The concentrations of Av1 and Av2 in the assay were

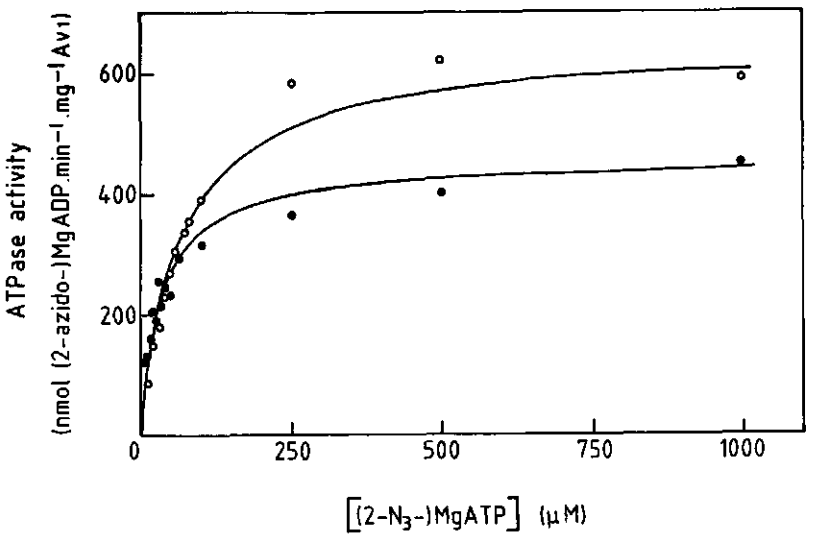

Fig. 1. *Concentration dependence of the reductant-independent hydrolysis rates of MgATP and 2-azido-MgATP*. Assay conditions are described under Materials and Methods. ATPase activities are expressed as nmol MgADP formed·min^{-1}·mg^{-1} Av1 (●-●), or as nmol 2-azido-MgADP formed·min^{-1}·mg^{-1} Av1 (O-O). Reaction temperature was 23 °C. The curves drawn through the data points are fits according to the Michaelis-Menten parameters given in the text.

0.4 μM and 2.0 μM respectively. From a double-reciprocal plot of the data points obtained with MgATP an apparent K_m of 35 μM and a V_{max} of 456 nmol MgADP formed$\cdot min^{-1}\cdot mg^{-1}$ Av1 were calculated. For 2-N_3-MgATP an apparent K_m and V_{max} of respectively 32 μM and 644 nmol 2-azido-MgADP formed$\cdot min^{-1}\cdot mg^{-1}$ Av1 were obtained. The tetrazolo tautomer of 2-N_3-MgADP is a poor substrate for pyruvate kinase [11]. As a consequence, the pyruvate kinase/lactate dehydrogenase assay monitors predominately the formation of the azido tautomer of 2-N_3-MgADP. Thus, if the tetrazolo tautomer of 2-N_3-MgATP is also a substrate for nitrogenase, the true value for V_{max} will higher. V_{max} will also be underestimated if the tetrazolo tautomer of 2-N_3-MgATP binds to the nitrogenase complex but is not hydrolysed. The true value for K_m will be lower than the number given here if the tetrazolo tautomer of 2-N_3-MgATP does not bind to the nitrogenase complex.

Effect on enzyme activities

Irradiation of Av2 (10 μM) in the presence of 2-N_3-MgADP (50 μM) lead to almost complete inactivation of the Fe protein as measured in the acetylene assay. The inactivation of Av2 was not prevented by the presence of 750 μM MgADP. Thus, Av2 must possess a binding site specific for the azido group. This conclusion is corroborated by the observation that 2-azido-MgAMP is equally effective in inactivating Av2. Fig. 2 summarizes the influence of the 2-N_3-MgAMP concentration on the catalytic activity of Av2 (after irradiation) as measured in the acetylene reduction, reductant-dependent, and reductant-independent ATPase activity assay. The same figure also summarizes the effect of labelling on the ratio mol ligated Fe:mol Av2. It is stressed here that the inactivation process of Av2 is accompanied by loss of iron from the [4Fe-4S] cluster. Addition of 750 μM MgAMP or MgADP was without significant effect on the degree of inactivation by 2-azido-MgAMP (50 μM). A control experiment

showed that prephotolyzed 2-N_3-MgAMP was ineffective in inactivating Av2 (not shown).

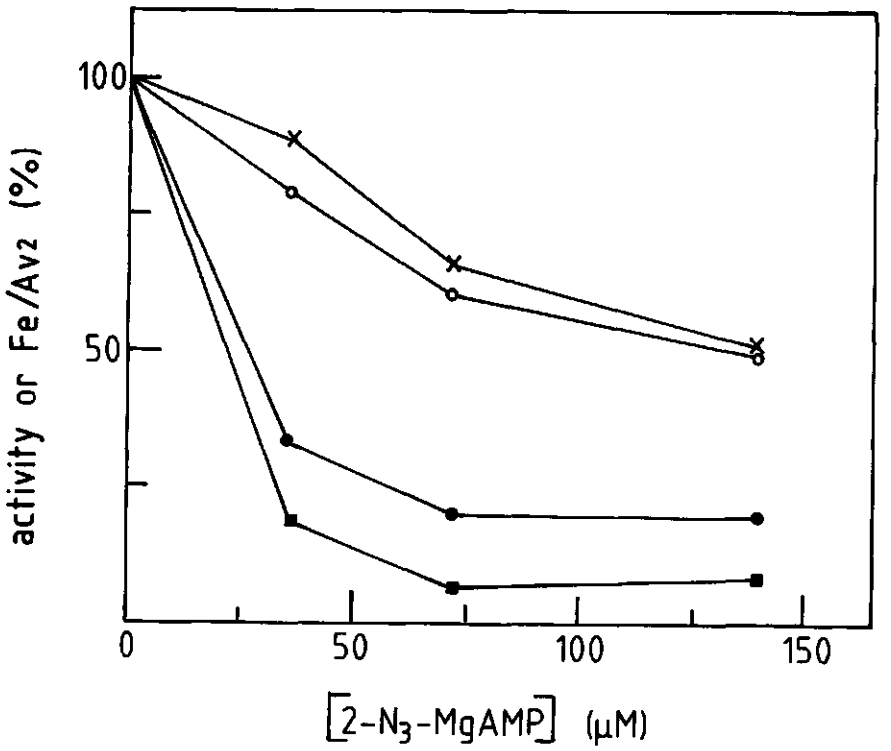

Fig. 2. *Concentration dependence of photoinactivation of Av2 by 2-azido-MgAMP.* Experiments have been performed as described under Materials and Methods. (■-■), nitrogenase activity; (●-●), reductant-dependent ATPase activity; (O-O), reductant-independent ATPase activity; (x-x), Fe:Av2. 100%-values are: nitrogenase activity, 1560 nmol C_2H_4 formed·min^{-1}·mg^{-1} Av2; reductant-dependent and reductant-independent ATPase activity, 10205 and 478 nmol ADP formed·min^{-1}·mg^{-1} Av2 respectively; ratio Fe:Av2 = 3.4.

Irradiation of Av1 (8 μM), or Ac1 (5 μM), in the presence of 2-N_3-MgADP (291 μM) had no significant effect on the nitrogenase or reductant-independent ATPase activity of the MoFe proteins. Also, the BPS reaction gave no indication that the metal-clusters of the MoFe proteins are decomposed by any reaction with the photoaffinity label.

Quantization and electrophoresis experiments

Av2 (11 μM) was irradiated in the presence of 2-N_3-[α-^{32}P]MgAMP (82 μM), 2-N_3-[α-^{32}P]MgADP (68 μM), and 2-N_3-[α-^{32}P]MgATP (82 μM). In order to detect specific competition for the nucleotide binding sites, the labelling experiments with 2-N_3-[α-^{32}P]MgADP and 2-N_3-[α-^{32}P]MgATP have also been performed in the presence of 1 mM MgAMP, MgADP, and MgATP. After irradiation labelled Av2 was precipitated and subjected to electrophoresis. In competition experiments the ratio label:Av2 has been determined prior and after electrophoresis.

Fig. 3 shows autoradiographs of Av2 samples labelled by the azido nucleotide analogues and the accompanying competition experiments. After electrophoresis the amount of 2-azido-[α-^{32}P]MgADP and 2-azido-[α-^{32}P]MgATP linked to Av2 is noticeable higher than that of the MgAMP analogue (Fig. 3A). As judged by autoradiography, labelling of Av2 with 2-azido-[α-^{32}P]MgADP is inhibited by MgADP and MgATP, but not by MgAMP (Fig. 3B). The same pattern is observed with 2-azido-[α-^{32}P]MgATP (Fig. 3C). These results prove that the 2-azido analogues of MgADP and MgATP label the nucleotide binding sites on the Fe protein.

In Table 1 the ratios label:Av2 before and after electrophoresis are compared. The data reveal that Av2 has lost label in each of the electrophoresis experiments. However, the residual amount of label is greater in the competition experiments with MgAMP. As the competition experiments with

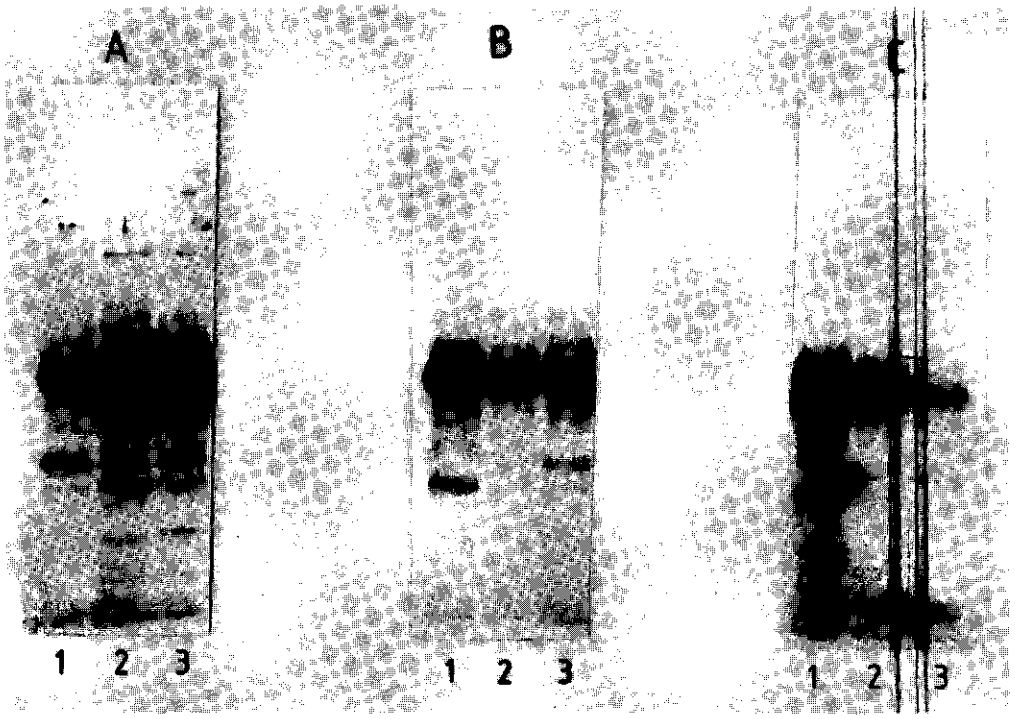

Fig. 3. *Autoradiographs of Av2 labelled by 2-azido-[α-^{32}P] analogues of adenosine phosphates*. Precipitated labelled nitrogenase proteins have been dissolved and electrophorized as described under Materials and Methods. Labelling conditions have been given in the text. The amount of Av2 applied on gel was either 146 picomol (A), or 186 picomol (B,C). (A) Av2 labelled by 2-azido-[α-^{32}P] analogues of respectively MgAMP (lane 1), MgADP (lane 2), and MgATP (lane 3). (B) Av2 labelled by 2-azido-[α-^{32}P]MgADP in the presence of MgAMP (lane 1), MgADP (lane 2), and MgATP (lane 3). (C) Av2 labelled by 2-azido-[α-^{32}P]MgATP in the presence of MgAMP (lane 1), MgADP (lane 2), and MgATP (lane 3).

MgAMP clearly demonstrate, the addition of 1 mM nucleotide provides also a degree of aspecific protection against labelling. This is probably due to a reduced labelling efficiency, caused by absorption of ultraviolet light by the unmodified nucleotides.

Table 1. *Release of label during gel electrophoresis*
Radioactivity in electrophorized protein samples was determined as described under Materials and Methods. Protein and nucleotide concentrations have been given in the text.

Sample	Ratio label:Av2	
	Before electrophoresis	After electrophoresis
Av2 + 2-N_3-MgAMP	0.20	n.d
+ 2-N_3-MgADP	0.32	n.d
+ 2-N_3-MgATP	0.27	n.d
Av2 + 2-N_3-MgADP + MgAMP	0.06	0.01
+ MgADP	0.04	<0.002
+ MgATP	0.09	<0.002
Av2 + 2-N_3-MgATP + MgAMP	0.12	0.01
+ MgADP	0.08	<0.002
+ MgATP	0.06	<0.002

n.d. = not determined.

The competition experiments with Av2 as described in the preceding paragraph have been repeated with Av1 and Ac1 (data not shown). No evidence was obtained for specific labelling by 2-azido-[α-^{32}P]MgADP or 2-azido-[α-^{32}P]MgATP.

Loss of label by modified Av2

The low molar ratios label:Av2 found in the protein bands of electrophorized samples indicate that the formed bond between label and protein is unstable in the electrophoresis protocol. We have tested the hypothesis that 2-mercaptoethanol affects the release of label. Table 2 summarizes a series of gel centrifugation experiments in which Av2 was labelled by 2-N_3-[α-^{32}P]MgAMP or 2-N_3-[α-^{32}P]MgADP and subsequently incubated with 2-mercaptoethanol. In the control experiments 2-mercaptoethanol was omitted. The results show that 2-mercaptoethanol is capable of releasing 2-azido-MgAMP and 2-azido-MgADP covalently bound to Av2.

Table 2. *Release of covalently bound 2-azido analogues of MgAMP and MgADP effected by incubation with 2-mercaptoethanol*
See Materials and Methods for the experimental procedure. The concentration of Av2 was 10 μM in each experiment. Nucleotide concentrations were: 167 μM 2-N_3-[α-^{32}P]MgAMP and 137 μM 2-N_3-[α-^{32}P]MgADP. Added mercaptoethanol was 5% (v/v).

Expt	Label	2-mercapto-ethanol	Ratio label:protein (mol:mol)
1.	2-N_3-[α-^{32}P]MgAMP	-	0.7
2.	2N_3-[α-^{32}P]MgAMP	+	0.1
3.	2-N_3-[α-^{32}P]MgADP	-	0.9
4.	2-N_3-[α-^{32}P]MgADP	+	0.2

Gel-filtration experiments

The nitrogenase component proteins were incubated with nucleotides and separated from the incubation mixture by gel-filtration. The separation took approximately 2 minutes. It was assumed that 2-N_3-[α-^{32}P]MgADP found in the protein fraction had been bound to a binding site characterized by a low rate of dissociation.

Table 3. Binding of 2-N_3-[α-^{32}P]MgADP to dye-oxidized nitrogenase proteins
Gel filtration experiments have been performed as described in Materials and Methods. The ratio label:protein in the effluent refers to the main protein fraction (± 0.5 ml). After irradiation this fraction was precipitated and washed, after which the ratio label:protein was again determined.

Expt	Starting concentrations			Ratio label:protein (mol:mol)	
	Protein (μM)	2-N_3-[α-^{32}P]MgADP (μM)	MgADP (mM)	In effluent	After irradiation and precipitation
1.	Av2 (225 μM)	567	-	0.25	0.01
2.	Av2 (208 μM)	523	3.8	<0.01	<0.01
3.	Av1 (77 μM)	213	-	<0.01	n.d
4.	Ac1 (47 μM)	213	-	0.10	0.08
5.	Ac1 (42 μM)	283	4.2	0.70	0.70

n.d. = not determined

Table 3 summarizes the gel-filtration experiments performed with 2-N_3-[α-^{32}P]MgADP. The molar ratio of 2-N_3-[α-^{32}P]MgADP to Av2 in the main protein fraction was equal to 0.25 (exp. 1). However, the elution profile showed that Av2 slowly releases 2-N_3-[α-^{32}P]MgADP during gel-filtration (not shown). MgADP competed successfully with 2-N_3-[α-^{32}P]MgADP for the slowly dissociating nucleotide site on Av2 (exp. 2). An insignificant amount of 2-N_3-[α-^{32}P]MgADP coeluted with Av1 (exp. 3). No competition experiment has been performed with Av1. Since Ac1 is the only MoFe protein of nitrogenase which has been reported to form a tight complex with MgADP [7], we have included this protein in the gel-filtration experiments. The ratio 2-N_3-[α-^{32}P]MgADP:Ac1 measured in the effluent was 0.10 (exp. 4). An unexpected observation was made in the experiment in which MgADP was meant to compete with 2-N_3-[α-^{32}P]MgADP for the slowly dissociating nucleotide site (exp. 5). Rather than suppressing the binding of 2-N_3- [α-^{32}P]MgADP, MgADP enhanced the amount of azido analogue coeluting with Ac1.

For all labelled samples it was found that electrophoresis resulted in loss of label. No preferential labelling of one of the subunits of the MoFe protein was evident from the autoradiographs (not shown).

DISCUSSION

It has been shown here that 2-azido-MgATP is a good substrate for nitrogenase in the reductant-independent ATPase assay. With an Av2:Av1 ratio of five, 2-azido-MgATP is hydrolysed at a greater rate than MgATP. Cordewener *et al.* [4] have proposed that in the catalytic cycle of the reductant-independent ATPase activity of nitrogenase the dissociation of MgADP from the Fe protein is the rate limiting step. According to this mechanism, a greater reaction rate would imply that 2-

azido-MgADP dissociates faster from Av2 than MgADP does. This could also explain why Av2 releases 2-azido-MgADP during a gel-filtration experiment.

Fig. 2 shows that the reductant-independent ATPase activity is less susceptible to inactivation by 2-azido-MgAMP than the substrate reducing activity and reductant-dependent ATPase activity. A similar observation has been reported for experiments with partially O_2-inactivated Av2 [19]. The reason at a molecular level for these observations is still unknown. Possibly, inactivation of the electron transferring capability of the Fe protein occurs in a single, for instance by removal of a single Fe atom from the [4Fe-4S] cluster. Inactivation of the reductant-independent ATPase activity may be a gradual process.

It was found that 2-azido-[α-^{32}P]MgADP, like MgADP [5], binds to a slowly dissociating site on dye-oxidized Ac1. This observation suggests that a tight nucleotide binding site on dye-oxidized Ac1 can be occupied by 2-azido-MgADP. Ac1 still binds 2-N_3-MgADP tightly if MgADP is present in excess. This is probably due to an affinity site for the azido group which is present in the complex between Ac1 and MgADP. The ratio label:Ac1 of 0.10 reported here is substoichiometric, which may be due to a non-saturating concentration 2-azido-[α-^{32}P]MgADP or release of label on the column. Despite the affinity of Ac1 for 2-azido-MgADP, the protein was not inactivated in labelling experiments.

The results obtained with Av2 prove that the 2-azido-analogues of MgADP and MgATP label the nucleotide binding sites on the Fe protein. It is also concluded that Av2 has a binding site which is specific for the azido-group. Labelling of this second site results in partial decomposition of the [4Fe-4S] cluster. Since the [4Fe-4S] cluster of Av2 is ligated to the conserved cysteines 97 and 132 [23], it is proposed here that these cysteines are labelled by the 2-azido-nucleotide analogues and released by reduction with 2-mercaptoethanol. This mechanism

has an analogy with a recent report on the labelling of a cysteine residue of phosphoenolpyruvate carboxykinase by 8-azido-GTP [24]. The labelled cysteine was shown to release label as the result of reduction by a neighbouring cysteine residue. Furthermore, cysteine 38 which has been suggested to be presented at the nucleotide binding site [23] might be a target for the 2-azido-analogues of MgADP and MgATP and release label during electrophoresis.

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CHAPTER 3

A REINVESTIGATION OF THE PRE-STEADY-STATE ATPase ACTIVITY OF THE NITROGENASE FROM *AZOTOBACTER VINELANDII*

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SUMMARY

The pre-steady-state ATPase activity of nitrogenase has been reinvestigated. The exceptionally high burst in the hydrolysis of MgATP by the nitrogenase from *Azotobacter vinelandii* communicated by Cordewener *et al.* [(1987) *Eur. J. Biochem.* 162, 265-270] was found to be caused by an apparatus artefact. A second possible artefact in the determination of the stoichiometry of the pre-steady-state ATPase activity of nitrogenase was observed. Acid quenched mixtures of either Av1 or Av2, and MgATP contained phosphate above the background level. It is proposed that due to this reaction quenched reaction mixtures of nitrogenase and MgATP may contain phosphate in addition to the phosphate released by the ATPase activity of the nitrogenase complex.

It was found feasible to monitor MgATP dependent pre-steady-state proton production by the absorbance change at 572 nm of the pH indicator o-cresolsulfonphthalein in a weakly buffered solution. At 5.6 °C, a pre-steady-state phase in H^+-production was observed with a rate constant of 1.5 s^{-1}, whereas electron transfer occurred with a rate constant of 5.1 s^{-1}. At 20.0 °C, MgATP dependent H^+-production and electron transfer were in the pre-steady-state phase characterized by rate constants of 11.1 s^{-1} and 99 s^{-1} respectively. The stopped-flow technique failed to detect a burst in the release of protons by the dye-oxidized nitrogenase complex.

It is concluded that the hydrolysis rate of MgATP as judged by proton release is lower than the rate of electron transfer from the Fe protein to the MoFe protein.

INTRODUCTION

Biological reduction of dinitrogen is catalysed by the enzyme complex nitrogenase. The enzyme complex comprises a MoFe protein and a Fe protein. Properties of the component proteins [1] and the current accepted model [2] for the nitrogenase reaction have been the subject in review articles.

For catalytic activity nitrogenase requires the presence of MgATP and a strong reductant. Experiments performed under steady-state conditions show that 2 MgATP are hydrolysed for each electron transferred to substrate [3]. By the rapid freeze EPR [4,5] and stopped-flow spectrophotometry technique [6,7] it was demonstrated that the presence of MgATP is a prerequisite for electron transfer from the Fe protein to the MoFe protein. Eady *et al.* [8] have published a combined stopped-flow spectrophotometry and rapid quench study in which the pre-steady-state electron transfer and MgATP hydrolysis was investigated. Both reactions were at 10 °C characterized by a burst phase which could both be fitted to a single exponential function. Since the rate constants were found to be identical within experimental error, these results were interpreted as evidence that electron transfer from the Fe protein to the MoFe protein and the ATPase activity are directly coupled. It was concluded that one mol MgATP was hydrolysed for each mol electron transferred. The authors proposed that an additional MgATP is hydrolysed in a second reaction step. Additional rapid quench studies confirmed a burst in the hydrolysis of MgATP, but no consensus was reached on the ratio mol MgATP hydrolysed per mol electron transferred. Hageman *et al.* [9] concluded that all MgATP is hydrolysed concomitant with the reduction of the MoFe protein. The data obtained by Cordewener *et al.* [10], and Mensink & Haaker [11] suggest that as many as 8 MgATP per mol Av1 can be hydrolysed in a burst reaction.

Recently, Thorneley *et al.* [12] have used the stopped-flow

calorimetry technique to monitor pre-steady-state heat consumption and proton production by the nitrogenase from *Klebsiella pneumoniae* after mixing with MgATP. It was concluded that the hydrolysis of MgATP precedes electron transfer from the Fe protein to the MoFe protein. However, no mechanism has been developed yet for the hydrolysis of MgATP and how the free energy of hydrolysis is utilized in nitrogenase catalysis.

The present report is the first of two papers in which data are presented of a reinvestigation of the role of MgATP in the catalytic cycle of nitrogenase. In this chapter the reliability of the rapid quench technique as a method of monitoring the pre-steady-state ATPase activity of nitrogenase is discussed. In the following chapter of this thesis the temperature dependence of the rate and extent of MgATP induced reduction of the MoFe protein is analyzed.

MATERIALS AND METHODS

Cell growth and enzyme preparation

Azotobacter vinelandii ATCC strain 478 was grown in a 200 litre fermenter on a modified, nitrogen free Burk's medium as described by Burgess *et al.* [13]. The growth curve was followed by monitoring the turbidity at 660 nm. Cells were harvested in the logarithmic phase. Component proteins were isolated and assayed by acetylene reduction as described by Braaksma *et al.* [14]. The molar concentration of Av1 and Av2 were calculated from the relative molecular masses of 220 kDa and 63 kDa respectively.

Av1 was oxidized with solid thionine as oxidant [15]. Av1(ox) and the excess thionine were separated on a Bio-Gel P-6DG column equilibrated with 0.5 mM Tes/NaOH buffer containing 10 mM $MgCl_2$ and 200 mM NaCl (to avoid precipitation of Av1),

pH 7.8. Av2 was oxidized with phenazine methosulfate as described elsewhere [16]. The Av2(ox) stock solution contained 0.5 mM Tes/NaOH and 10 mM $MgCl_2$, pH 7.8.

Dithionite free nitrogenase complex was prepared by running a concentrated mixture of Av1 and Av2 in the required ratio over a Bio-Gel P-6DG column (1x10 cm) equilibrated with 0.5 mM Tes/NaOH, 10 mM $MgCl_2$, pH 7.8. Total protein concentration of the eluent was determined by the microbiuret method. The dithionite free nitrogenase complex was used immediately after preparation.

The specific activities of the Av1 and Av2 preparations were at least 2800 and 1800 nmol ethylene produced$\cdot min^{-1}\cdot mg^{-1}$. protein respectively. Av1 contained 1.8±0.2 mol Mo/mol Av1. The Fe content of Av2 was 3.6±0.3 mol Fe/mol Av2.

Analytical methods

Protein concentrations were determined by the microbiuret method of Goa [17] after a precipitation step with deoxycholic acid and trichloric acid [18]. Bovine serum albumin was used as a standard. At least three quantities of the samples were used for each determination. Molybdenum [19], iron [20], and orthophosphate [21] were determined by slightly modified published procedures.

Chemicals

ATP (special quality) and cresol red (sodium salt) were obtained from Boehringer and Sigma respectively. 2,4-dinitrophenyl acetate was supplied by Eastman Kodak.

Instrumentation

The rapid quench apparatus, model 150/151, was purchased

from Update Instrument, Inc (Madison, USA). Aging hoses were made of nylon and varied in length between 10 and 210 cm; the internal diameter was 0.5 mm. The original syringes have been replaced by 2.5 ml Hamilton (gastight 1002) syringes, since the original construction of the syringes with o-rings results in a backflow of reactants (see below). As an additional precaution the mixing chamber was disassembled after each push and cleaned. Also, the first 100 µl were pushed out of the hoses which connect the syringes and mixing chamber.

One syringe contained the nitrogenase proteins (concentrations as indicated) in 50 mM Tes/NaOH, 10 mM $MgCl_2$, final pH 7.4. The other syringe contained 13 mM ATP, 50 mM Tes/NaOH, 13 mM $MgCl_2$, final pH 7.4. Sodium dithionite concentration in both syringes was 10 mM. Data points between 17 and 482 milliseconds were produced in the single push mode. Background orthophosphate in each syringe has been determined in a sample taken at the start and end of the experiment. The averaged values have been used to calculate background P_i (µM) in the reaction mixture. Syringes were mounted in a argon saturated thermostated water bath. All rapid quench experiments were performed at either 10.0±0.5 °C or 22.0±0.5 °C.

A Hi-TECH SF-51 stopped flow spectrometer (Salisbury, Wilts, U.K.) equipped with an anaerobic kit, data acquisition and analysis system has been used to observe the pre-steady-state electron transfer from Av2 to Av1. The oxidation of Av2 was monitored at 430 nm. Syringe A contained Av1 (20 µM), Av2 (20-320 µM), 10 mM sodium dithionite, 10 mM $MgCl_2$, in 50 mM Tes/NaOH, final pH 7.4. Syringe B contained 13 mM ATP, 10 mM sodium dithionite in 50 mM Tes/NaOH, 13 mM $MgCl_2$, final pH 7.4. The progression curves for MgATP induced oxidation have been monitored between 2 and 6 times. Final data were calculated from the averaged curves.

Detection of released protons

Protons liberated by the reaction between MgATP and the nitrogenase complex were monitored in a stopped-flow spectrophotometer as the absorbance change at 572 nm of the pH indicator o-cresolsulfonphthalein (cresol red). The technique was essentially that described by Finlayson & Taylor [22].

One syringe contained 12-14 μM Av1, 80-134 μM Av2, 10 mM $MgCl_2$, 50 μM sodium dithionite, in 0.5 mM Tes/NaOH, final pH 7.8. The second syringe contained 4 mM ATP, 4 mM $MgCl_2$, and 100 μM cresol red in argon saturated 0.5 mM Tes/NaOH, pH 7.8. Dithionite was omitted in experiments with dye-oxidized nitrogenase proteins. Experiments have been performed at either 5.6±0.1 °C or 20.0±0.1 °C.

The buffer capacity of the reaction mixtures were estimated by titrating a mixture of the nitrogenase proteins (concentrations as indicated) with the exception that ADP was replaced by ATP. One millilitre mixture was transferred into an argon flushed cuvette capped with a Subaseal closure. The cuvette was placed in an Aminco 2a spectrophotometer. Argon saturated HCl (10 mM) was added in portions of 2 μl to 8 μl to titrate the mixture. Absorbance changes were monitored at 572 nm.

RESULTS

Performance of the rapid quench apparatus

Results which indicated that the nitrogenase from *A. vinelandii* hydrolyses 8 MgATP/mol Av1 have been published by this laboratory [10,11]. In these experiments samples for the determination of background phosphate were drawn directly from the syringes. In a sequel to these studies blanks were taken

after each produced data point. In order to collect these blanks the tubing which connects the syringes and mixing chamber were disconnected at the mixing chamber and reactants were pushed through the tubing. Phosphate determinations revealed that these blanks contained 15 to 30 nmol more phosphate than those taken directly from the syringes. This observation suggested that backflow of mixed reactants had occurred after each push. The occurrence of such a backflow could be directly visualized by mixing a phenolphthalein solution (5 mg/ml BSA added) with a 5 mM NaOH solution. A pink streak, formed by mixing of both solutions, was clearly visible over a length of 2 to 5 millimetres in the nylon hoses (internal diameter 1 mm) which connect the syringes with the mixing chamber. The backflow was not observed if the syringes supplied by Update were replaced by 2.5 ml Hamilton (gastight 1002) syringes.

Performance of the modified rapid quench apparatus has been assessed by measuring the rate constant for the base catalysed hydrolysis of 2,4-dinitrophenylacetate. At 22 °C and with a NaOH concentration equal to 0.25 M (after mixing) a second order rate constant of 58 $M^{-1} \cdot s^{-1}$ was obtained, which is in good agreement with the value of 52.4 $M^{-1} \cdot s^{-1}$ (at 23 °C) reported by Froehlich *et al.* [23].

Prior to the reinvestigation of the pre-steady-state ATPase activity of nitrogenase, the minimal Av2:Av1 ratio (Av1=10 μM after mixing) necessary to reduce all Av1 present was determined. Both at 10 °C and 22 °C this ratio was found to be 5.

The colorimetric phosphate assay has been tested under reaction conditions similar to those employed in the study on the pre-steady-state ATPase activity of the nitrogenase complex. Table 1 summarizes a number of tests in which BSA, Av1, and Av2 have been mixed with MgATP. The test reaction with BSA reveals that no MgATP is hydrolysed as a consequence of mixing, or by any other mechanism inherent to the construction of the rapid

Table 1. *Acid induced release of inorganic phosphate by MgATP in the presence of Av1 or Av2*
The separate component proteins of nitrogenase or BSA were mixed with MgATP (final concentration 6.5 mM) and quenched in 10% (w/v) TCA. Protein concentrations after mixing were: 5 mg/ml BSA, 14 μM Av1 and 30 μM Av2. Background P_i/ml reaction mixture in the experiments with BSA, Av1, and Av2 was 15±2 μM, 16±4 μM, and 16±4 μM respectively. Reaction temperature was 22 °C.

	nmol P_i above background, per ml reaction mixture		
Reaction time (ms)	BSA	Av1	Av2
27	-3	14	14
70	0	16	17
136	3	15	16
214	4	19	21
291	-2	11	14
350	1	24	14

quench apparatus. In contrast, acid quenched reaction mixtures of the separate nitrogenase proteins and MgATP contain inorganic phosphate well above the background.

We have attempted to determine the reaction progression curve of the pre-steady-state ATPase activity of reduced nitrogenase. Experiments have been performed at 10 °C in order to obtain data points within the resolution time of the rapid quench apparatus. A set of four rapid quench experiments with saturating Av2 concentrations has been summarized in Figure 1. Though the data in Figure 1 show a rise in the concentration P_i

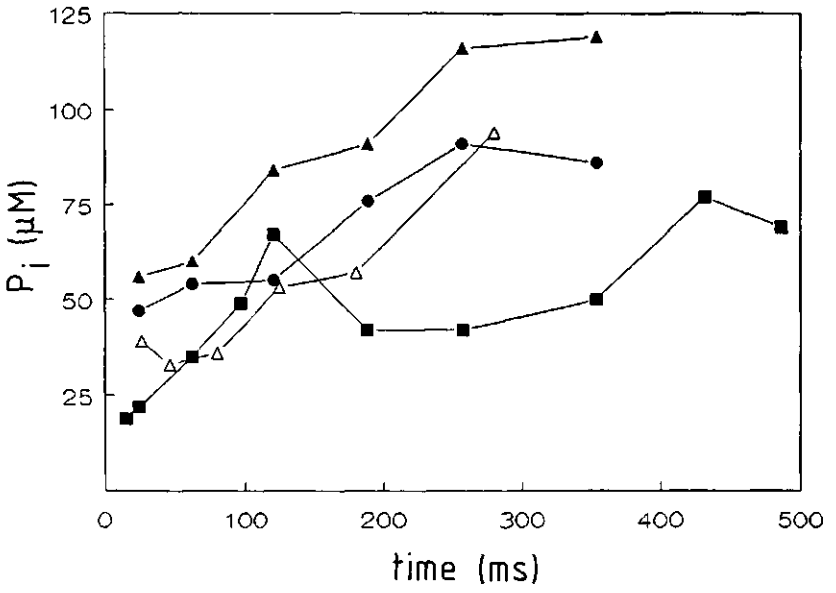


Fig. 1. *Appearance of inorganic phosphate in acid quenched mixtures of nitrogenase and MgATP.* Experiments have been performed at 10 °C. Experimental conditions and the phosphate assay have been described under Materials and Methods. Background phosphate (b.g. P_i) has been subtracted from the amount orthophosphate found in the quenched reaction mixture. Protein concentrations are after mixing. (■-■), 20 μM Av1 and 120 μM Av2, b.g. P_i 25±20 μM; (Δ-Δ), 20 μM Av1 and 120 μM Av2, b.g. P_i 6±1 μM; (●-●), 20 μM Av1 and 120 μM Av2, b.g. P_i 9±1 μM; (▲-▲), 22 μM Av1 and 219 μM Av2, b.g. P_i 8±1 μM.

with time, it is obvious that the scatter in the data points do not allow a detailed kinetic analysis. It should also be noted here that, despite the fact that background phosphate present in the protein and MgATP solution has been subtracted, the curves

do not extrapolate to 0 μM P_i formed at zero reaction time. Though, an upper limit for the amount of P_i formed has been roughly estimated by taking the difference between the orthophosphate concentration at t=17 ms (first data point) and the maximum P_i concentration observed at a later reaction time. At 10 °C the observed rate constant for electron transfer was 12 s^{-1}, whereas the absorbance change associated with the MgATP dependent oxidation of the Fe protein was found to be 70% of the maximal value ($\Delta\epsilon(430)$= 5.5±0.6 $mM^{-1} \cdot cm^{-1}$). It can thus be calculated that 0.7x(36±4)= 25±3 μM FeMoco has been reduced in the pre-steady-state phase. The combined data give a stoichiometry of 2.1±0.3 (n=4) P_i formed/FeMoco reduced.

Liberation of protons by nitrogenase

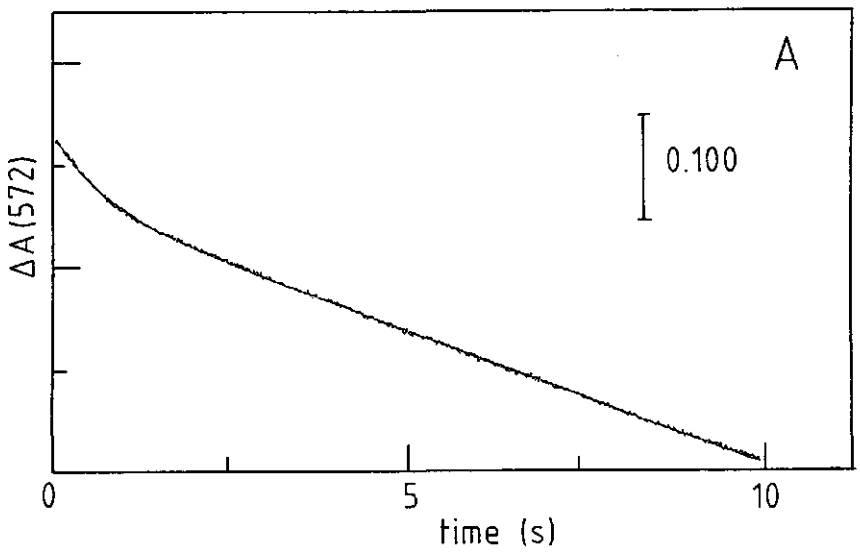
To our knowledge, all pH indicators which can in principle be used to monitor the production of protons in the reaction of nitrogenase with MgATP, are bleached by millimolar concentrations dithionite. This reaction is likely to interfere with attempts to monitor the production of protons by nitrogenase under these conditions. However, stopped-flow experiments (not shown) demonstrated that at 5.6 °C and 20.0 °C bleaching of 50 μM cresol red by 25 μM dithionite in 0.5 mM Tes/NaOH, pH 7.8 gives an absorbance change of $6x10^{-4}$ s^{-1} and $4x10^{-3}$ s^{-1} respectively. So, the reaction of protons with cresol red can be monitored in the presence of 25 μM dithionite, provided the absorbance change associated with proton production is large compared to the latter values. The absorbance change in the absence of dithionite was less than $1x10^{-4}$ s^{-1}.

The buffer capacity of the used nitrogenase solutions were estimated as described under Materials and Methods. Data points were averaged to give a value for $\Delta A(572)/\Delta[H^+]_{added}$. A significant observation is that the latter value is lowered by the presence of NaCl, see Table 2 for a summary.

Table 2. *Buffer capacity of nitrogenase/MgADP solutions*
Data were obtained as described under Materials and Methods. In each experiment the solution contained 0.5 mM Tes/NaOH, 7 mM $MgCl_2$, 2 mM ADP, 50 μM cresol red, final pH 7.8. $\Delta A(572)/\Delta[H^+]_{added}$ (M^{-1}) and the standard error have been calculated from 4 data points.

Exp.	Mixture	$\Delta A(572)/\Delta[H^+]_{added}$ (M^{-1})
1	Buffer + ADP	6500 ± 900
2	Buffer + ADP + 50 mM NaCl	3800 ± 400 (Lowered by NaCl)
3	Buffer + Av1 (6 μM) + Av2 (40 μM) + ADP	3100 ± 200
4	Buffer + Av1(ox) (7 μM) + Av2(ox) (67 μM) + ADP + 50 mM NaCl	1400 ± 150 (Lowered by NaCl)

The reaction between cresol red and H^+ has been used to monitor proton release as the result of the reaction between MgATP and nitrogenase. Experiments have been carried out at 5.6 °C and 20.0 °C. At both reaction temperatures it was observed that mixing Av2 with the MgATP/cresol red solution resulted in a small absorbance change (not shown). This absorbance change has been subtracted from the progression curves obtained with the nitrogenase complex. The rates of electron transfer given below, have been obtained by mixing the nitrogenase complex with MgATP in the absence of cresol red. At 5.6 °C the reaction rate of electron transfer was found to be 5.1 s^{-1}. No proton production with a larger reaction rate was evident. However, proton production was detected on a longer time scale (see Fig. 2A). The progression curve for proton production fits to $\Delta A(572) = 0.070 \exp(-1.5t) - 0.026t$. The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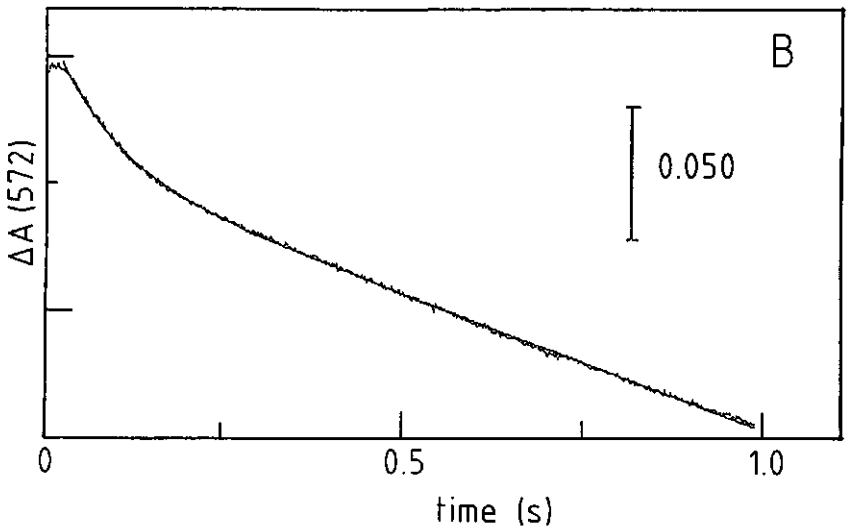
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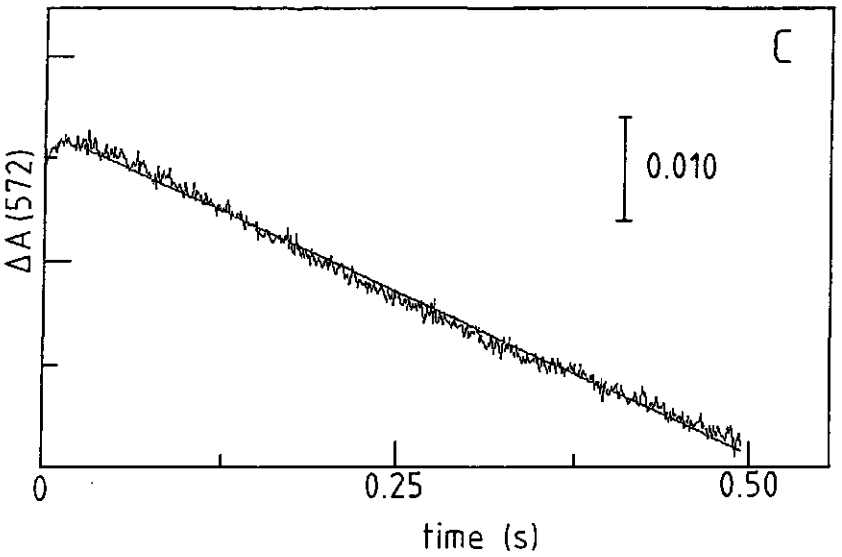

Fig. 2. *Production of protons in the catalytic cycle of nitrogenase*. The change in pH was monitored as the absorbance change of cresol red in a weakly buffered reaction mixture as described under Materials & Methods. Protein concentrations are after mixing. Opposing page: (A) [Av1]=6 μM and [Av2]=42 μM at 5.6 °C; (B) [Av1]=6.5 μM and [Av2]=40 μM at 20.0 °C. Above: (C) [Av1(ox)]=7 μM [Av2(ox)]=67 μM at 20.0 °C. The protein syringe contained 50 μM sodium dithionite in the experiments with reduced nitrogenase.

relevant conclusion can be drawn here that at the described reaction conditions the nitrogenase complex displays a pre-steady-state phase in which protons are produced with a lower rate than the MgATP induced oxidation of the Fe protein. The burst phase in Fig. 2A agrees with 3.8 mol $H^+ \cdot mol^{-1}$ Av1. The

linear phase corresponds with 380 nmol H^+ formed·min^{-1}·mg^{-1} Av1.

At 20.0 °C the rate constant for MgATP dependent electron transfer was equal to 99 s^{-1}. The absorbance change due to the reaction of cresol red with protons liberated by the nitrogenase complex fits to $\Delta A(572) = 0.054 \exp(-11.1t)-0.107t$ (see Fig. 2B). The amplitude of the fitted curve corresponds with 2.7 mol H^+·mol^{-1} Av1. It should be noted here that this stoichiometry is obtained by extrapolating the fitted curve to zero reaction time. If the plateau in the progression curve represents a real maximal value the stoichiometry becomes 1.7 mol H^+·mol^{-1} Av1. The linear phase agrees with 1450 nmol H^+ formed·min^{-1}·mg^{-1} Av1.

MgATP dependent proton production by the dye-oxidized nitrogenase complex has been measured at 20.0 °C with Av1(ox)=7 μM and Av2(ox)=67 μM, see Fig. 2C. It is evident that on a time scale of 0.5 second no burst in H^+-production is observed. The initial absorbance change illustrated in Fig. 2C corresponds with 1700 nmol H^+ formed·min^{-1}·mg^{-1} Av1(ox).

All the absorbance changes summarized in Figure 2 were absent if the experiment was repeated in 100 mM Tes/NaOH, pH 7.8. This observation rules out that other processes than proton production contribute to the monitored absorbance change.

DISCUSSION

A major problem in attempts to rationalize the mechanism by which electron transport and MgATP hydrolysis are coupled has been the value of the ratio mol MgATP hydrolysed/mol MoFe protein reduced in the pre-steady-state phase. Eady *et al.* [8] concluded that 1 mol MgATP per mol Kp1 is hydrolysed concomitant with electron transfer. However, the authors have been unaware of the fact that the amplitude of the stopped-flow signal which accompanies MgATP induced oxidation of the Fe protein is

temperature dependent [12,24]. The data obtained with the nitrogenase from *K. pneumoniae* by Thorneley *et al.* [12] show that at 6 °C approximately 50% of the Kp1 is reduced. It is thus possible that Eady *et al.* [8] have overestimated the amount of Kp1 which was reduced in their experiment and that the ratio mol MgATP hydrolysed/mol Kp1 reduced has been close to 2.

The primary data of Hageman *et al.* [9] indicate that in their experiments 1 mol MgATP/mol Av1 has been hydrolysed. After applying a correction for inactive protein based on the Mo content of Av1 and the reaction of the [4Fe-4S] cluster in Av2 with bathophenanthrolinedisulfonate, the authors conclude that the ratio mol MgATP hydrolysed/mol Av1 is equal to 2. It should be noted here that Hageman *et al.* [9] have used an Av2:Av1 of 2.5. Results obtained in our laboratory with the nitrogenase from *A. vinelandii* show that at 20.0 °C this ratio is suboptimal and gives approximately 50% of the maximal absorbance change (obtained with Av2:Av1≥5). It is thus likely that only 50% of the FeMo-cofactor could be reduced, which implies that the data of Hageman *et al.* [9] are consistent with a ratio mol MgATP hydrolysed/mol electron transferred of 2.

It is also noteworthy that published kinetic data on the pre-steady-state ATPase activity have not been entirely in concord with a model in which electron transfer is triggered by the hydrolysis of MgATP. Thorneley *et al.* [12] concluded from their experimental results that at 6 °C the hydrolysis of MgATP precedes electron transfer. The progression curves for the pre-steady-state ATPase activity obtained at 22 °C [10] and 30 °C [9] have not been fitted to a single-exponential function. However, visual inspection of these curves makes it clear that these progression curves have a half life-time of approximately 50 milliseconds, which corresponds with a first order rate constant of 20 s^{-1}. This rate constant is much slower than the rate constant for electron transfer (larger than 100 s^{-1}) at those reaction temperatures.

The construction of a reliable progression curve with the aid of the rapid quench technique requires that the separate nitrogenase proteins are inert with respect to mixing with MgATP, followed by quenching in acid. In our laboratory we found this not to be the case and assume that this phenomena is likely to interfere with attempts to determine inorganic phosphate produced by the hydrolysis of MgATP by the nitrogenase complex. It is concluded here that this artefact and the described backflow, invalidate the rapid quench data reported by Cordewener *et al.* [10] and Mensink & Haaker [11]. Unfortunately, no data are available to judge whether the same conclusion is valid for the results obtained by Hageman *et al.* [9] with an identical rapid quenching apparatus which introduced in our case backflow of reactants.

The rapid quench data published by Eady *et al.* [8] reveal at 10 °C P_i production with a reaction rate close to that of electron transfer. Using the cresol red/stopped-flow spectrophotometry technique, we failed at 5.6 °C and 20.0 °C to detect proton release prior to electron transfer. It is suggested here that the acid quench is responsible for phosphate liberation at the greater reaction rate. A possible mechanism is that at any reaction temperature electron transfer induces a strain in the P_γ-OP_β bond, which leaves the phosphate bond susceptible to hydrolysis by acid.

In this study the ratio mol H^+ liberated·mol^{-1} Av1 at 20 °C was found to be significantly lower than 4. This number indicates that, if one accepts that 4 MgATP are hydrolysed in the first cycle of the nitrogenase complex, less than 2 protons are released per electron transferred. This suggests that 2 protons remain bound to the nitrogenase complex.

A study on the steady-state ATPase activity by dye-oxidized nitrogenase as measured in an ATP regenerating system has been published by Cordewener *et al.* [25]. According to their data a mixture with Av2(ox):Av1(ox)=9.6 gives an ATPase activity

of approximately 900 nmol MgADP·min^{-1}·mg^{-1} Av1(ox). The initial steady-state ATPase activity of 1700 nmol H^+ formed·min^{-1}·mg^{-1} Av1(ox) (Av2:Av1=9.6) reported in the current paper is significantly higher. Since Cordewener *et al.* [25] have used an ATP regenerating system, it is suggested that their ATPase assays have been partly inhibited by MgADP.

In conclusion, the data presented here show that as judged by the stopped-flow/cresol red technique, the hydrolysis of MgATP by the nitrogenase complex is slower than electron transfer from the Fe protein to the MoFe protein. However, our results disagree with data published by Thorneley *et al.* [12], who found with the stopped-flow microcalorimetry technique that at 6 °C the nitrogenase from *K. pneumoniae* liberates protons with a faster rate than electron transfer. At present we have no explanation for the discrepancy between the data of Thorneley *et al.* [12] and those presented in this paper. A more detailed investigation is required in which data obtained by both the stopped-flow calorimetry technique [12] and cresol red/stopped-flow spectrophotometry (this work) are compared.

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CHAPTER 4

TEMPERATURE EFFECTS ON THE MgATP INDUCED ELECTRON TRANSFER BETWEEN THE NITROGENASE PROTEINS FROM *AZOTOBACTER VINELANDII*

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SUMMARY

The temperature dependence of the pre-steady-state MgATP dependent electron transfer from the MoFe protein to the Fe protein of the nitrogenase from *Azotobacter vinelandii* has been investigated between 6 °C and 31 °C by stopped-flow spectrophotometry. Below 14 °C the data are consistent with a model in which interaction of MgATP with nitrogenase is fast and irreversible, and is followed by reversible electron transfer. The rate constant for reverse electron flow was found to be approximately 4 s^{-1} and invariant with the reaction temperature. Analysis of the data obtained in the temperature range between 6 °C and 12 °C within the framework of the transition-state theory show that electron transfer from the Fe protein to the MoFe protein occurs via a highly disordered transition-state with activation parameters $\Delta H^{\circ\ddagger}$=289 $kJ \cdot mol^{-1}$ and $\Delta S^{\circ\ddagger}$=792 $J \cdot K^{-1} \cdot mol^{-1}$. The Eyring plot of the stopped-flow data displays an inflection point around 14 °C. From the stopped-flow data obtained between 18 °C and 27 °C the activation parameters $\Delta H^{\circ\ddagger}$ and $\Delta S^{\circ\ddagger}$ for the reduction of the MoFe protein are calculated to be 90 $kJ \cdot mol^{-1}$ and 99 $J \cdot K^{-1} \cdot mol^{-1}$ respectively. A second inflection point in the Eyring plot is evident around 28 °C.

The positive values for the activation entropies associated with MgATP dependent electron transfer from Av2 to Av1 indicate that electron transfer occurs via a highly disordered transition state.

INTRODUCTION

Nitrogenase is composed of a MoFe protein and a Fe protein. The presence of both proteins, a low potential electron donor and MgATP are necessary for the biological reduction of dinitrogen to ammonia [1]. A detailed model for the kinetics of nitrogen reduction by the nitrogenase complex has been developed. In this model the hydrolysis of 2 MgATP are coupled to electron transfer from the Fe protein to the MoFe protein [2,3]. Support for this mechanism is generally derived from two investigations [3,4] on the pre-steady-state ATPase activity of the nitrogenase complex. Recently, Thorneley *et al.* [5] concluded from data obtained in a combined stopped-flow spectrophotometry and stopped-flow calorimetry study that electron transfer from Kp2 to Kp1 is preceded by the hydrolysis of MgATP and that electron transfer from the Fe protein to the MoFe protein is a reversible process.

In several communications the effect of temperature on nitrogenase catalysis has been used to gain information on the mechanism of action of the nitrogenase complex. The Arrhenius plot for the association constant of the nitrogenase complex from *Klebsiella pneumoniae* exhibits a break around 17 °C [6]. It is also known that the ATP:e value for the nitrogenase from *A. vinelandii* is affected by reaction temperature, with a minimum value of 2 around 20 °C [7,8]. Both effects have been explained by assuming a temperature induced conformation change of the nitrogenase complex.

In this paper the results of an investigation of the temperature dependence of the extent and rate of MgATP dependent electron transfer from the Fe protein to the MoFe protein are presented. The current paper supplements the preceding communication [9] in which the detection of a possible burst in the hydrolysis of MgATP by nitrogenase has been reinvestigated. The implications of the combined results for the current model

of MgATP dependent electron transfer from the Fe protein to the MoFe protein are discussed.

MATERIALS AND METHODS

The nitrogenase component proteins from *Azotobacter vinelandii* ATCC 478 were purified and assayed as described elsewhere [10]. Protein concentrations were estimated by the microbiuret method [11] after a precipitation step with deoxycholic acid and trichloric acid [12]. Molybdenum [13] and iron [14] were determined by published methods which have been slightly modified. Av1 and Av2 preparations had at 30 °C specific activities of 2300-2800 and 1900-2100 nmol ethylene produced·min^{-1}·mg^{-1} protein respectively. Av1 and Av2 contained respectively 1.8±0.2 mol Mo/mol Av1 and 3.6±0.3 mol Fe/mol Av2.

Stopped-flow measurements were performed with a HI-TECH SF51 stopped-flow apparatus (Salisbury, Wilts, U.K.) equipped with an anaerobic kit, data acquisition and analysis system. Stopped-flow traces have been fitted to a single exponential function. The dead reaction time has been calculated by comparing the absorbance change due to the total bleaching of 2,4-dichloro-indophenol by ascorbic acid as measured in a Perkin-Elmer Lambda 1A spectrophotometer with the amplitude of the progression curve of the same reaction mixture monitored by the stopped-flow spectrophotometer. The dead reaction time was found to be 1.5 ms and has been taken into account in the determination of signal amplitudes.

Reactants were dissolved in 50 mM Tes/NaOH, pH 7.4. The protein solution contained 20 μM Av1, 140 μM Av2, 10 mM $MgCl_2$, and 5 mM $Na_2S_2O_4$. The second syringe contained 13 mM ATP, 13 mM $MgCl_2$, and 5 mM $Na_2S_2O_4$.

Computer simulations were performed with the KINSIM program written and distributed by B.A. Barshop [15].

RESULTS AND DISCUSSION

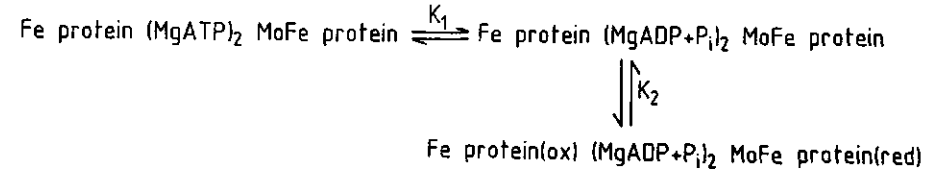
Temperature effect on the absorbance change

Thorneley et al. [5] have noticed that the absorbance change caused by the MgATP dependent pre-steady-state electron transfer from Kp2 to Kp1 is affected by the reaction temperature. Our data of the effect of reaction temperature on ΔA(430) and the observed rate of MgATP dependent electron transfer within the nitrogenase complex from *A. vinelandii* are summarized in Table 1.

In order to explain their data, Thorneley *et al.* [5] proposed a mechanism in which MgATP hydrolysis and electron

Table 1. *Data on the effect of the reaction temperature on the extent and observed rate of MgATP dependent electron transfer* Experiments have been performed as described under Materials and Methods. Standard error in ΔA(430) and k_{obs} is approximately 2%. K_2, k_2, and k_{-2} have been calculated according to Scheme 2 as explained in the text.

Temperature (K)	ΔA(430)	K_2	k_{obs} (s^{-1})	k_2 (s^{-1})	k_{-2} (s^{-1})
279.2	0.027	0.4	5.0	1.4	3.6
281.0	0.039	0.7	7.1	2.9	4.2
283.0	0.063	1.9	13.1	8.6	4.5
285.1	0.077	3.9	21.9	17.4	4.5
286.8	0.086	7.8	29.8	26.4	3.4
289.7	0.095		55		
291.5	0.091		77		
294.1	0.098		118		
295.8	0.098		153		
298.2	0.097		201		
299.2	0.100		196		
300.1	0.096		233		
301.1	0.103		235		
302.9	0.097		273		
304.6	0.088		293		



Scheme 1. *Current model of the kinetics of pre-steady-state MgATP hydrolysis and electron transfer within the nitrogenase complex.* Adapted from Thorneley *et al.* [5].

transfer are at 5 °C reversible, whereas these reactions are essentially irreversible at 23 °C, see Scheme 1. The magnitudes of K_1 and K_2 determine the fraction of the MoFe protein which is reduced by the Fe protein. It is demonstrated in Appendix A that according to Scheme 1 the absorbance change due to the oxidation of the Fe protein and the equilibrium constants K_1 and K_2 are related as follows:

$$\frac{\Delta A_{max}(430) - \Delta A(430)}{\Delta A(430)} = \frac{1}{K_2}\left\{1 + \frac{1}{K_1}\right\} \qquad (1)$$

In the Appendix it is also demonstrated that a plot of the natural logarithm of the left side of Eqn. (1) versus 1/T (K^{-1}) should curve in the temperature range where K_1 changes from a value of approximately 1 to a value larger than 10 as proposed by Thorneley *et al.* [5]. It is clear from Fig. 1 that no such effect is evident. Only data points in the range between 6.0-13.6 °C have been used, since at higher temperatures $\Delta A(430)$ approaches $\Delta A_{max}(430)$ with the result that the error in the left side of Eqn. (1) becomes unacceptable. The value used for $\Delta A_{max}(430)$ was 0.097.

Further support for the absence of a reversible reaction

which precedes electron transfer is obtained from an analysis of the stopped-flow signal. According to Thorneley *et al.* [5] the hydrolysis of MgATP precedes at 5 °C oxidation of the Fe protein. This conclusion has been based on the observation that the reaction of the nitrogenase complex from *K. pneumoniae* with MgATP as observed in a stopped-flow calorimetry experiment could be fitted to a single exponential function with a rate constant of 9 s^{-1}, whereas electron transfer was found to occur with a first order rate constant of 3 s^{-1}. In general, the reaction

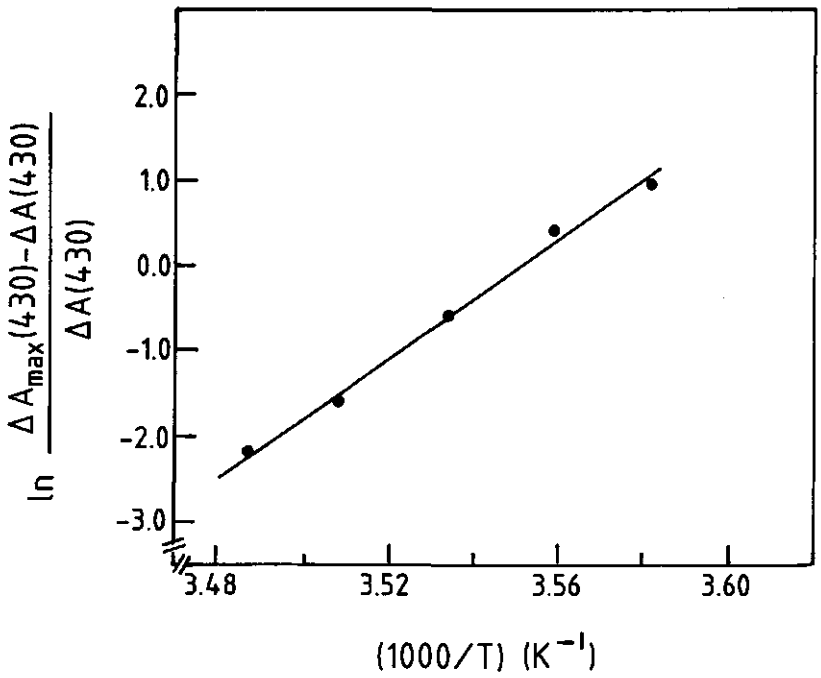


Fig. 1. *Effect of the reaction temperature on the extent of MgATP induced electron transfer from Av2 to Av1.* Experiments were performed as described under Materials and Methods. Data are presented according to equation (15) as explained in the Appendix.

progression curve of the last species being formed in two consecutive reactions may display an induction period. Whether this induction period is actually observed depends on the magnitude of the individual rate constants. We have resorted to computer modelling to assess whether the observations of Thorneley *et al.* [5] can be simulated. A successful simulation should for the endothermic reaction give a progression curve which fits to a single exponential function with a rate constant of 9 s^{-1}. At the same time, the progression curve which corresponds with the oxidation of the Fe protein should represent a single exponential progression curve with a rate constant of 3 s^{-1} and reflect the observation that 50% of the MoFe protein is reduced in the MgATP dependent electron transfer. The best simulations (not shown) to Scheme 1 were obtained with values which centred around k_1=9 s^{-1}, k_{-1}=9 s^{-1} (K_1=1), k_2=3.6 s^{-1}, and k_{-2}=1.8 s^{-1} (K_2=2). The simulated curves for electron transfer fitted to a single exponential function only after 300 ms. In addition, the simulated progression curves are marked by an induction period of about 50 milliseconds. No induction period has been reported by Thorneley *et al.* [5]. In Fig. 2A & 2B it is shown that at 7.8 °C (where $\Delta A(430) \sim 0.4\ \Delta A_{max}(430)$) an induction period is also absent in the oxidation of the Fe protein of *A. vinelandii*.

Changing the dithionite concentration from 5 mM to 0.5 mM at 10 °C and 23 °C was without effect on the reaction rate and observed absorbance change (not shown). This excludes the possibility that the lower extinction observed at low reaction temperatures is caused by a fast re-reduction of the Fe protein.

It is concluded here that Scheme 1 can not explain the decreased absorbance change associated with MgATP induced electron transfer from Av2 to Av1 by assuming that K_1 is reversible and of the same order of magnitude as K_2. In addition, it has been demonstrated in the preceding paper [9] that still no conclusive evidence is available which provides

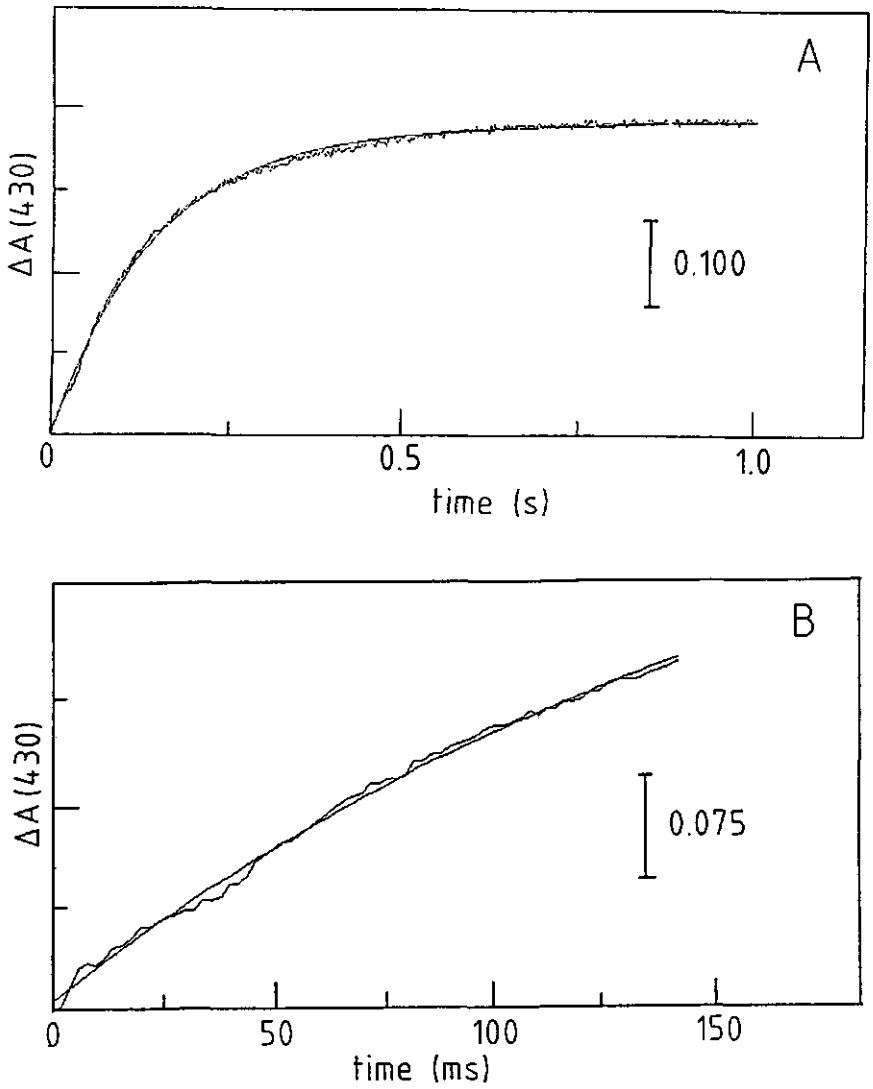


Fig. 2. *Progression curve of the MgATP induced electron transfer from Av2 to Av1.* The stopped-flow trace has been recorded as described under Materials and Methods. (A) Oxidation of Av2 as observed at 7.8 °C. (B) Detail of Fig. 2A. In both figures the progression curves fit to a single exponential function with a rate constant of 7.1 s^{-1}.

firm experimental evidence that hydrolysis of MgATP precedes electron transfer from the Fe protein to the MoFe protein of the nitrogenase complex. We therefore prefer to denote the MgATP which has 'energised' the nitrogenase complex as MgATP*. No commitment is made at this stage on the degree to which MgATP* has been hydrolysed. Thus, with fast and irreversible interaction of MgATP with nitrogenase the observable kinetics of electron transfer can be schematized as shown in Scheme 2.

$$\mathrm{Av2(MgATP^*)_2Av1} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \mathrm{Av2(ox)(MgATP^*)_2Av1(red)}$$

Scheme 2.

Temperature effect on the rate of electron transfer

In Scheme 2 the extent of MgATP induced electron transfer is determined by:

$$K_2 = k_2/k_{-2} \qquad (2)$$

Also, the observed rate of electron transfer is given by:

$$k_{obs} = k_2 + k_{-2} \qquad (3)$$

Combining Eqns. (2) and (3) gives the following expression for k_2:

$$k_2 = \frac{K_2}{1 + K_2} \; k_{obs} \qquad (4)$$

K_2 can be calculated from the absorbance change at 430 nm according to:

$$K_2 = \frac{\Delta A(430)}{(\Delta A_{max}(430)-\Delta A(430))} \qquad (5)$$

The calculated values for K_2, k_2, and k_{-2} in the temperature range between 6.0 °C and 13.6 °C have been listed in Table 1. The data in Table 1 show that according to Scheme 2 the reaction rate, k_{-2}, for the reverse reaction is independent of the reaction temperature.

The transition-state theory [16,17] states that each of the rate constants is related to the reaction temperature by:

$$k = (k_B T/h)\ e^{(\Delta S^{\circ\ddagger}/R)}\ e^{(-\Delta H^{\circ\ddagger}/RT)} \qquad (6)$$

In which k_B is the Boltzmann constant; T (K) the reaction temperature; h Planck's constant; R the gas constant; $\Delta S^{\circ\ddagger}$ the standard entropy of activation; $\Delta H^{\circ\ddagger}$ the standard enthalpy of activation.

Fig. 3 gives an Eyring plot (ln (k/T) vs 1000/T) for k_2. For reaction temperatures higher than 13.6 °C, it has been assumed that temperature invariant reverse electron flow is still present and can be estimated at 4 s^{-1}. Inflection points are manifest around 14 °C and 28 °C. Fig. 3 has been used to calculate enthalpies and entropies of activation. The results, obtained by linear regression analysis, have been summarized in Table 2. In both temperature ranges positive value for $\Delta S_2^{\circ\ddagger}$ are obtained. An unexpected result of the analysis within the framework of Scheme 2 is the outcome that k_{-2} is invariant with the reaction temperature. The activation entropy, $\Delta S_{-2}^{\circ\ddagger}$, for reversed electron flow is estimated to be negative by a large value (-232 $J \cdot K^{-1} \cdot mol^{-1}$). Thus, the pathway for reversed electron flow seems to require a minimal activation enthalpy and a highly unfavourable activation entropy (corresponding with an ordered transition-state).

In general, a positive activation entropy is

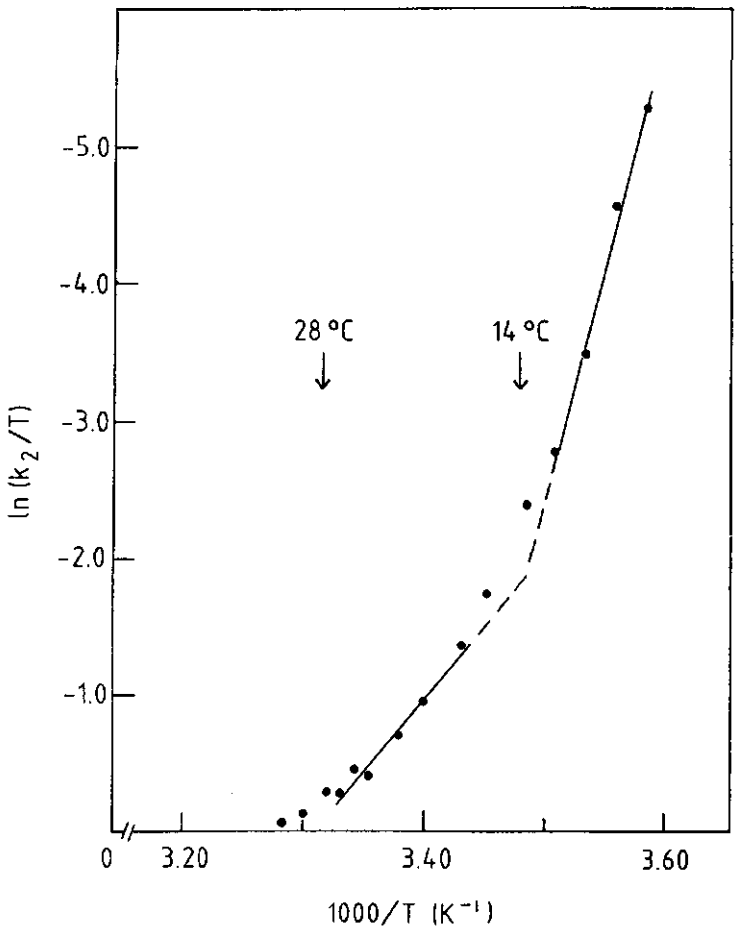


Fig. 3. *Influence of the reaction temperature on the rate of MgATP induced electron transfer from Av2 to Av1.* Eyring plot for the rate (k_2) of MgATP dependent electron transfer from Av2 to Av1.

characteristic for an enzyme-substrate complex which has undergone a major conformational change in the transition state,

Table 2. *Summary of enthalpies and entropies of activation* Parameters have been calculated as described in the text from the temperature dependence of the extent and rate of electron transfer from MgATP dependent electron transfer from Av2 to Av1.

	k_2		k_{-2}	
Temperature range (°C)	$\Delta H_2^{\circ\ddagger}$ kJ·mol^{-1}	$\Delta S_2^{\circ\ddagger}$ J·K^{-1}·mol^{-1}	$\Delta H_{-2}^{\circ\ddagger}$ kJ·mol^{-1}	$\Delta S_{-2}^{\circ\ddagger}$ J·K^{-1}·mol^{-1}
6.0 - 11.9	289	792	0	-232
18.3 - 26.9	90	99	-	-

possibly by release of protein bound water or by an increased mobility as the result of broken hydrogen-bridges [16]. Below 14 °C the activation entropy for electron transfer from Av2 to Av1 is exceptionally high and falls in the range of activation entropies found for protein denaturation [16]. It is suggested here that this observation should be explained by assuming that below 14 °C the nitrogenase complex exists in a rigid conformation and that a major rearrangement of the peptide chains has occurred in the transition-state. This structural alteration may be identical to the temperature induced conformational change of the nitrogenase complex which has been proposed before [6-8].

The activation parameters found for the temperature range between 18 °C and 27 °C are in Table 3 compared with two examples which involve reduction of a metalloprotein and are characterized by a positive activation entropy. In each of the examples a disordering in the mantle of protein bound water has been proposed as an explanation for the decreased ordering of the transition-state complex. The same explanation may be put forward here to explain the positive activation entropy for the

Table 3. *Comparison of the transition-state of nitrogenase with other electron transferring reactions involving metalloproteins* Only examples characterized by a positive activation entropy have been listed.

Reaction	Enthalpy of activation ($kJ \cdot mol^{-1}$)	Entropy of activation ($J \cdot mol^{-1} \cdot K^{-1}$)	Reference
MgATP induced reduction of Av1 by Av2 between 18 °C and 27 °C	90	99	This work
Reduction of plastocyanin by cytochrome f	43.9	46.0	Ref. 18
Reduction of $Co(phen)_3^{3+}$ by plastocyanin	58.6	20.9	Ref. 19

electron transfer from Av2 to Av1. Probably, the inflection point around 28 °C is caused by a second alteration in the conformation of the nitrogenase complex.

In the preceding paper [9] evidence has been presented which shows that the rate of MgATP dependent proton release by the nitrogenase complex is lower than the rate of electron transfer from Av2 to Av1. It is proposed here that binding of MgATP to the nitrogenase complex facilitates the formation of a transition-state which allows electron transfer. Actual hydrolysis of MgATP with the concomitant release of inorganic phosphate in the solvent may be obligatory for dissociation of a structurally altered nitrogenase complex which is formed as a consequence of electron transfer.

APPENDIX

According to Scheme (1) the absorbance change at 430 nm, $\Delta A(430)$, caused by the MgATP dependent oxidation of Av2:

$$\Delta A(430) = (\text{fraction super-reduced Av1})\ \Delta A_{max}(430) \tag{7}$$

The fraction super-reduced Av1, f(Av1(red)), is given by

$$\frac{[Av1(red)Av2(ox)(MgADP+P_i)_2]}{[Av1Av2(MgATP)_2] + [Av1Av2(MgADP+P_i)_2] + [Av1(red)Av2(ox)(MgADP+P_i)_2]} \tag{8}$$

With

$$K_1 = \frac{[Av1Av2(MgADP+P_i)_2]}{[Av1Av2(MgATP)_2]} \tag{9}$$

and

$$K_2 = \frac{[Av1(red)Av2(ox)(MgADP+P_i)_2]}{[Av1Av2(MgADP+P_i)_2]} \tag{10}$$

The fraction of super-reduced Av1 can be written as:

$$f(Av1(red)) = \frac{K_1K_2}{1 + K_1 + K_1K_2} \tag{11}$$

So that

$$\Delta A(430) = \frac{K_1K_2}{1 + K_1 + K_1K_2}\ \Delta A_{max}(430) \tag{12}$$

Eqn. (12) can be rearranged to give

$$\frac{\Delta A_{max}(430) - \Delta A(430)}{\Delta A(430)} = \frac{1}{K_2}\left\{1 + \frac{1}{K_1}\right\} \qquad (13)$$

A temperature dependence can be introduced into Eqn. (13) by noticing that the effect of temperature on the electron transferring reaction is related to

$$K_2 = e^{(-\Delta G_2^{\circ}/RT)} \qquad (14)$$

Substitution of Eqn. (14) in (13), writing ΔG° as $\Delta H^{\circ}-T\Delta S^{\circ}$, taking the natural logarithms, and some rearrangement gives

$$\ln\left\{\frac{\Delta A_{max}(430) - \Delta A(430)}{\Delta A(430)}\right\} = \left\{\frac{\Delta H_2^{\circ}}{RT} - \frac{\Delta S_2^{\circ}}{R}\right\} + \ln(1/K_1 + 1) \qquad (15)$$

If it is realized here that the equilibrium constant K_1 is also affected by the reaction temperature it is evident that a plot of the left handed side of Eqn. (15) versus (1/T) (K^{-1}) should curve in the temperature range where K_1 is approximately equal to 1.

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CHAPTER 5

REDUCTION OF THE FeMo-COFACTOR OF NITROGENASE FROM *AZOTOBACTER VINELANDII* AT A LOW REACTION TEMPERATURE AND HIGH NaCl CONCENTRATIONS

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SUMMARY

The effect of high NaCl concentrations on the MgATP dependent, pre-steady-state electron transfer from the Fe protein to the MoFe protein of the nitrogenase from *Azotobacter vinelandii* has been studied by stopped-flow spectrophotometry and rapid-freeze EPR spectroscopy. It was found that NaCl lowers the absorbance change at 430 nm which accompanies pre-steady-state electron transfer between the nitrogenase proteins. Rapid-freeze EPR experiments indicate that the decreased absorbance change as observed in the presence of 500 mM NaCl (at 23 °C) and at a reaction temperature of 6 °C can not be explained by assuming partial reduction of the MoFe protein. The implications of these results are discussed with respect to the current model for electron transfer between the Fe and MoFe proteins of the nitrogenase complex.

INTRODUCTION

Nitrogenase is the enzyme complex which catalyses the reduction of dinitrogen to ammonia. The nitrogenase complex comprises two metalloproteins. The largest (M_r ±220 kDa) is a tetramer ($\alpha_2\beta_2$). Each $\alpha\beta$-unit represents a catalytically independent moiety which contains one iron-molybdenum cluster (FeMo-cofactor, FeMoco) and one 8-Fe centre [1]. The FeMo-cofactor is thought to be the catalytic site for substrate reduction. The second component protein of nitrogenase is a homodimer (γ_2, M_r ±63 kDa). For catalytic activity nitrogenase requires the presence of a strong reductant and MgATP. Relevant biophysical properties of the nitrogenase proteins [2] and the catalytic cycle [3] have been reviewed.

Electron transfer from the Fe protein to the MoFe protein can be monitored by the stopped-flow spectrophotometry [4,5] or the rapid-freeze EPR technique [6,7]. At low reaction temperatures the absorbance change due to the MgATP induced oxidation of the Fe protein is diminished [8,9]. Thorneley *et al.* [8] have suggested that this effect can be explained by assuming that both hydrolysis of MgATP and electron transfer between the component proteins of nitrogenase are reversible processes. However, Mensink & Haaker [9] have argued that experimental evidence indicate that only electron transfer can be regarded as a reversible reaction.

Several studies have been conducted on the effects of high salt concentrations on the properties and catalytic activity of the nitrogenase complex. The $s_{20,w}$ value for the MoFe protein from *A. vinelandii* is increased by the presence of NaCl [10]. Binding of NaCl to the Fe protein from *A. vinelandii* is revealed by inhibition of the MgATP dependent chelation of the iron-sulphur cluster [11]. Salts are also known to be inhibitory in steady-state substrate reduction assays for the nitrogenase complex [10,11]. A cross-linking study with the nitrogenase

complex from *A. vinelandii* established that NaCl inhibits the association of the component proteins [12]. Deits & Howard [11] have developed a comprehensive kinetic model for the inhibitory effect of NaCl on substrate reduction by nitrogenase.

We report here that NaCl suppresses the absorbance change associated with MgATP dependent pre-steady-state electron transfer from Av2 to Av1. Rapid-freeze EPR data will be presented which indicate that this effect can not be attributed to incomplete reduction of the FeMo-cofactor. The significance of the results will be discussed with reference to possible reversible electron transfer between the nitrogenase proteins.

MATERIALS AND METHODS

The nitrogenase component proteins from *Azotobacter vinelandii* ATCC 478 were purified and assayed as described elsewhere [13]. Specific activities of Av1 and Av2 were 2300-2800 and 1900-2100 nmol ethylene produced$\cdot$min$^{-1}\cdot$mg^{-1} protein, respectively. Av1 contained 1.8±0.2 Mo/mol Av1. The iron content of the Fe protein was 3.6±0.3 mol, Fe/mol Av2.

Protein concentrations were estimated by the microbiuret method [14] after a precipitation step with deoxycholic acid and trichloric acid [15]. Molybdenum [16] and iron [17] were determined by slightly modified published procedures.

Stopped flow spectrophotometry has been performed with a HI-TECH SF51 stopped-flow apparatus (Salisbury, Wilts, U.K.) as described elsewhere [13]. In calculating the absorbance changes a dead reaction time of 1.5 ms was taken into account. The protein solution contained Av1 and Av2 in 50 mM Tes/NaOH, 10 mM $MgCl_2$, pH 7.4. Protein concentrations are given in the legends to the figures. The second syringe contained 13 mM ATP and 13 mM $MgCl_2$ in 50 mM Tes/NaOH, pH 7.4. Both syringes contained 5 mM sodium dithionite.

Rapid-freeze experiments were performed at 23 °C with the modified rapid-mixing apparatus from Update Instrument, Inc (Madison, USA) apparatus described elsewhere [13]. The end of the aging hose was connected to a nozzle from which the reaction mixture sprayed into an funnel to which an EPR tube was connected with a rubber tubing. Both the funnel and EPR tube were emersed in an isopentane/N_2(l) mixture. The formed 'snow' was packed in the EPR tube and kept in liquid nitrogen for further analysis by EPR spectroscopy. EPR spectra were obtained with a Bruker EPR-200 D spectrometer, with periferal instrumentation and data acquisition as described elsewhere [18]. EPR conditions used were: microwave frequency, 9.32 GHz; microwave power, 8.0 mW; modulation frequency, 100 kHz; modulation amplitude, 1.0 mT; temperature 15 K. The concentration super-reduced FeMoco was calculated from the decrease in the amplitude of the S=3/2 feature at g=3.64. The decrease in the double integrated S=1/2 signal (g=2.06, 1.94, and 1.85) was used to estimate the concentration of oxidized Av2. Blanks were obtained by mixing a nitrogenase solution with buffer in which ATP was omitted.

RESULTS AND DISCUSSION

The effect of the Av2:Av1 ratio on ΔA(430) in the presence of 250 mM NaCl, at 23 °C, is illustrated in Fig. 1. The maximal value for ΔA(430) is obtained at an Av2:Av1 ratio of 6, which is equal to the ratio found in the absence of NaCl (not shown). It must thus be concluded that NaCl has no effect on the ratio redox active Fe protein/redox inactive Fe protein. The absorbance change with Av2:Av1>6 corresponds with ΔA(430)= 3.2±0.3 mM^{-1} Mo·cm^{-1}. This values differs significantly from the value of 5.5±0.3 mM^{-1} Mo·cm^{-1} which is obtained in the absence of NaCl [9]. In the presence of 250 mM NaCl, the observed rate

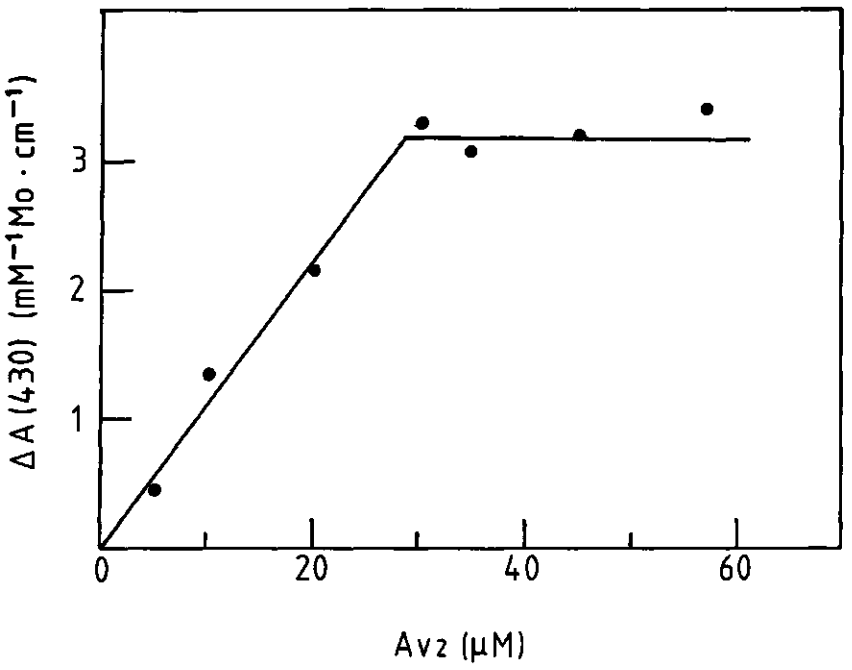


Fig. 1. *Titration of the amplitude of the stopped-flow signal with Av2 at a constant Av1 concentration in the presence of 250 mM NaCl.* Experimental conditions have been described under Materials and Methods. Av1 concentration after mixing was 5 μM. Both syringes contained 250 mM NaCl. Reaction temperature was 23 °C.

constants increased from 23 s^{-1} to 34 s^{-1} with increasing Av2:Av1 ratio (data not shown). This effect must probably be attributed to a raise in the fraction of Av1 which is complexed to $Av2(MgATP)_2$.

The effect of NaCl on the amplitude of the stopped-flow signal has been summarized in Fig. 2A. A Hill plot for the effect of NaCl on the observed rate of MgATP dependent electron

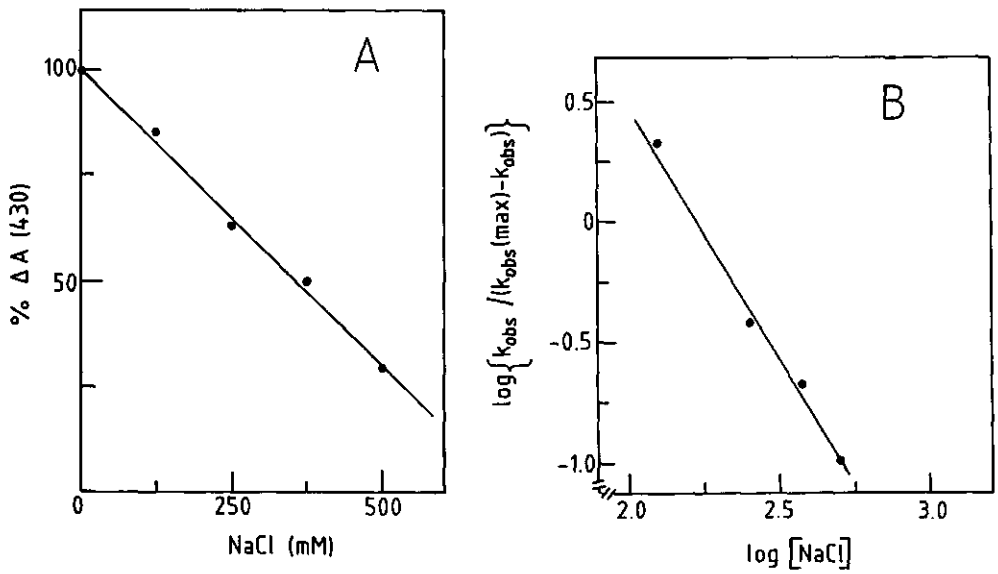


Fig. 2. *Effect of the NaCl concentration on the absorbance change and observed reaction rate*. Protein concentrations after mixing: [Av1]=10 μM, [Av2]=60 μM. Reaction temperature was 23 °C. (A) Absorbance change caused by MgATP dependent electron transfer from Av2 to Av1. (B) Hill plot for the observed rate of MgATP dependent electron transfer.

transfer is given in Fig. 2B. The data fit to a Hill coefficient of 2.1, which indicates that the inhibition of NaCl on the observed rate of electron transfer occurs in a cooperative fashion. The amplitude of the stopped-flow signal as a function of reaction temperature in the absence and presence of 250 mM NaCl has been summarized in Fig. 3. Maximal values for the absorbance change in the absence and presence of 250 mM NaCl are 5.3±0.3 mM^{-1} Mo·cm^{-1} and 5.5±0.3 mM^{-1} Mo·cm^{-1} respectively.

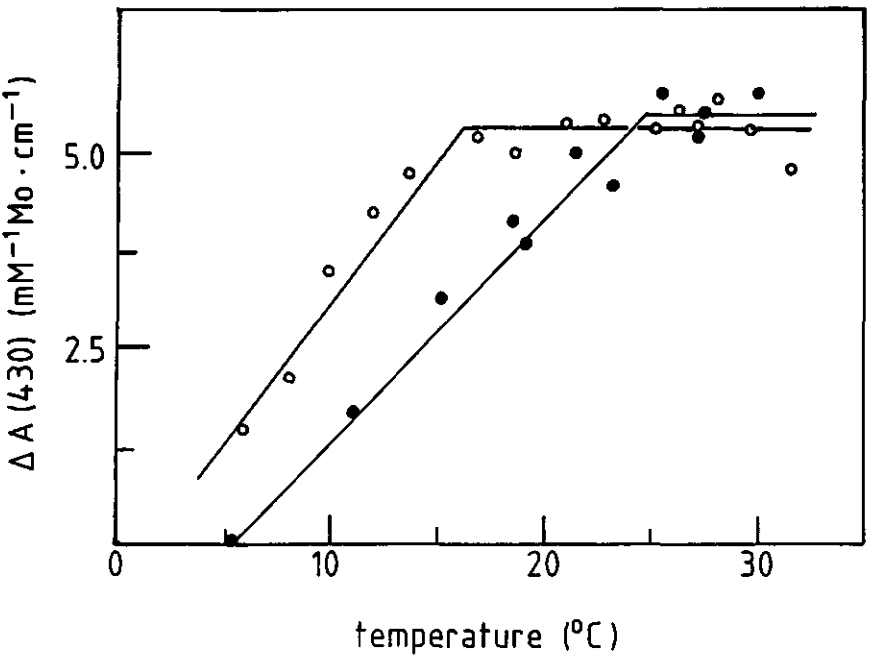


Fig. 3. *Effect of reaction temperature on the absorbance change.* Reaction conditions are given under Materials and Methods. Open symbols is in the absence of NaCl (O-O); closed symbols is in the presence of 250 mM NaCl (●-●). Protein concentrations after mixing are: [Av1]=10 μM, [Av2]=60 μM. No absorbance change was observed in the presence of 250 mM NaCl at a reaction temperature of 5.2 °C.

However, the temperature at which the maximal value for ΔA(430) is realized, has been shifted from approximately 16 °C to 25 °C.

Stopped-flow experiments in which both syringes contained 250 mM NaCl, or 500 mM NaCl was added to either the MgATP or the protein solution generated identical traces (not shown). From

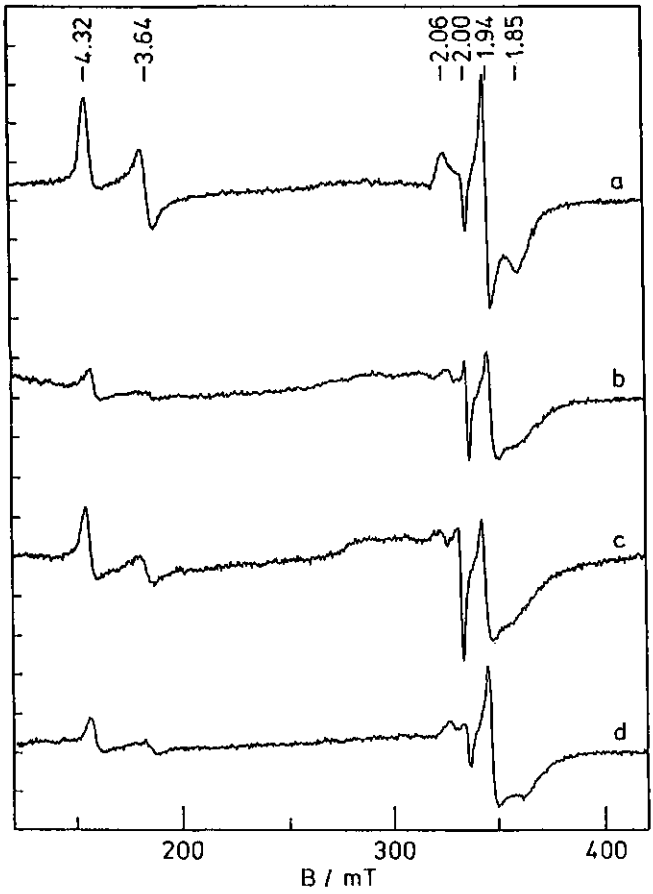


Fig. 4. *Reduction of FeMoco and oxidation of Av2 observed with the rapid-freeze EPR technique*. Rapid-freeze samples have been obtained as described under Materials and Methods. Protein concentrations after mixing: [Av1]=20 μM, [Av2]=120 μM. Trace a is a blank for experiment b and serves as an illustration for the EPR features of a blank nitrogenase sample. The features given by traces c and d have been quantified relative to separate blanks (not shown). Trace b) reaction at 23 °C, reaction time 30 ms; trace c) reaction at 6 °C, reaction time 1 s; trace d) reaction at 23 °C in the presence of 500 mM NaCl, reaction time 300 ms.

Table 1. *Comparison of the redox-state of FeMoco as determined by stopped-flow spectrophotometry and EPR rapid-freeze spectroscopy*

Data were obtained as described under Materials and Methods. In stopped-flow spectrophotometry experiments concentrations after mixing were: Av1=10 μM (18 μM Mo) and Av2=60 μM. In rapid-freeze experiments concentrations after mixing were: Av1=20 μM (36 μM Mo) and Av2=120 μM.

	Stopped flow	EPR integration	
Condition	amplitude ΔA(430)	super-reduced FeMoco	oxidized Av2
No NaCl, 23 °C (30 ms)	100%	34±5 μM (95%)	35±25 μM
No NaCl, 6 °C (1 s)	50%	28±5 μM (80%)	20±25 μM
500 mM NaCl, 23 °C (300 ms)	30%	31±5 μM (90%)	30±25 μM

these observations it must be inferred that the effect of NaCl is complete within the mixing time of the stopped-flow spectrophotometer. This implies that in the model of Deits & Howard [11] binding of NaCl to the nitrogenase complex and the subsequent dissociation of the component proteins must be fast.

The EPR rapid-freeze technique has been used to check whether reversible electron transfer is manifested in the redox-state of the nitrogenase proteins (Fig. 4). See Table 1 for a quantisation of super-reduced FeMoco and oxidized Av2. From the results it is evident the partial reduction of FeMoco as predicted from stopped-flow spectrophotometry is not confirmed

by rapid-freeze EPR measurements.

At this stage it is informative to construct an Eyring plot with the data from Chapter 4 with the assumption that electron transfer from the Fe protein to the MoFe protein is not

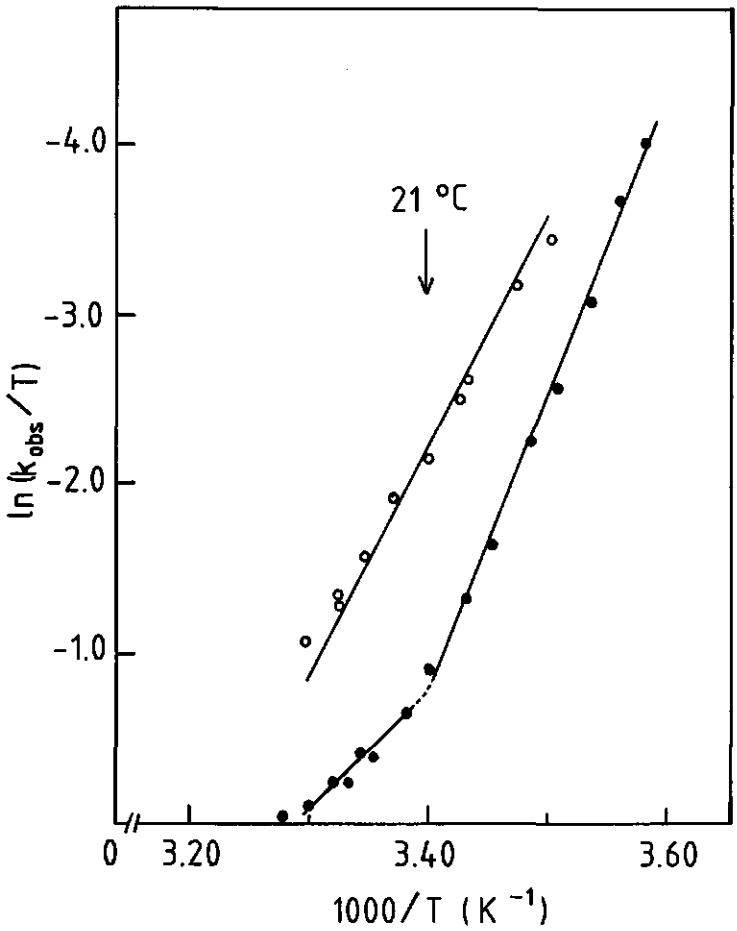


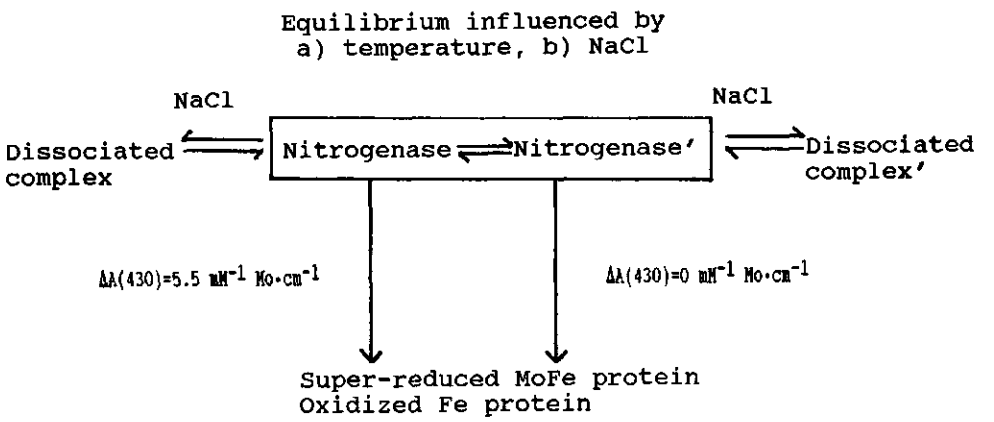
Fig. 5. *Eyring plots for MgATP dependent electron transfer from Av2 to Av1 with the assumption of irreversible electron transfer.* In the presence of 250 mM NaCl (O-O). In the absence of NaCl (●-●) (data points from Chapter 4, table 1).

reversible but irreversible. Thus, k_{obs} is the first order rate constant for electron transfer from the Fe protein to the MoFe protein. The recalculated Eyring plot has been illustrated in Fig. 5. The same figure shows the Eyring plot for the observed rate constants of MgATP dependent electron transfer in the presence of 250 mM NaCl as obtained in the current study. This exercise gives for electron transfer in the absence of NaCl the activation parameters $\Delta H^{\circ\ddagger}$=145 kJ·mol^{-1} and $\Delta S^{\circ\ddagger}$=288 J·mol^{-1}·K^{-1} for the temperature range between 6.0 °C and 20.9 °C. The Eyring plot displays an inflection point around 21 °C. For the temperature range between 22.6 °C and 29.7 °C the activation parameters are $\Delta H^{\circ\ddagger}$=57 kJ·mol^{-1} and $\Delta S^{\circ\ddagger}$=-11 J·mol^{-1}·K^{-1} respectively. Hence, also with the assumption of irreversible electron transfer the activation entropy remains highly positive for reaction temperatures below 21 °C and can still be interpreted as evidence for a highly disordered transition state. At reaction temperatures above 21 °C the high activation entropy is lost, probably due to a conformation change in the nitrogenase complex.

Figure 5 also shows that in the presence of 250 mM NaCl a linear Eyring plot is obtained. This suggests that the conformation change in the nitrogenase complex around 21 °C as observed in the absence of NaCl, has not occurred within the studied temperature range. From the Eyring plot the activation parameters $\Delta H^{\circ\ddagger}$=111 kJ·mol^{-1} and $\Delta S^{\circ\ddagger}$=163 J·mol^{-1}·K^{-1} can be calculated. It should be noted that the latter parameters contain contributions from the electron transferring reaction and any equilibrium which is introduced by the addition of NaCl. Since no information is available on the temperature dependence of these equilibria, it is not feasible to estimate the magnitude of possible effects of NaCl on the activation parameters for MgATP dependent electron transfer within the nitrogenase complex.

The data presented here are of special interest since they

challenge the necessity to introduce reversible electron transfer between the nitrogenase proteins as reported elsewhere [8,9]. An alternative explanation has been outlined in Scheme 1. It is proposed here that the nitrogenase complex can assume a conformation (primed in Scheme 1) in which MgATP dependent electron transfer is not accompanied by an absorbance change. A possible model for this phenomena has been presented in the General Discussion of this thesis. The equilibrium is shifted towards the latter conformation by lowering the reaction temperature or by the addition of NaCl. It is not necessarily implied here that the action of NaCl on this equilibrium requires binding to one of the nitrogenase proteins. The equilibrium could also be sensitive to the dielectric constant of the reaction medium. Secondly, the effect of NaCl on the observed reaction rate for electron transfer is ascribed to an inhibition of complex formation.



Scheme 1. *Alternative explanation for the suppression of the absorbance change associated with electron transfer from the Fe protein to the MoFe protein.*

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CHAPTER 6

GENERAL DISCUSSION

In 1967 it became firmly established that nitrogenase hydrolyses MgATP during turnover [1]. The molecular mechanism by which MgATP fulfils its function was still obscure when the work presented in this thesis was initiated. In fact, any speculation in the literature on the function of MgATP focuses on two observations:

a) Under optimal steady-state conditions the nitrogenase complex hydrolyses 2 MgATP for each electron which is transferred to substrate [2];

b) Rapid-quench experiments indicated that pre-steady-state electron transfer from the Fe protein to the MoFe protein and hydrolysis of MgATP both occur on a time scale of the same magnitude [3-5].

The ATP:e ratio of 2 which is obtained obtained under steady-state conditions is not contended. However, the literature is in disagreement on the ratio ATP:e ratio for electron transfer from the Fe protein to the MoFe protein in the pre-steady state phase of the nitrogenase reaction. The work described in this thesis was aimed at gaining information on the mechanism of action of MgATP in the pre-steady-phase of the nitrogenase reaction.

6.2 *Affinity labelling of the nitrogenase proteins*

In Chapter 2 of this thesis experiments are described which were aimed at characterizing affinity sites for the

photolabels 2-N_3-MgADP and 2-N_3-MgATP on Ac1, Av1, and Av2.

It could be demonstrated by gel-filtration and labelling experiments that the 2-azido analogues of MgADP and MgATP bind to the nucleotide site on dye-oxidized Av2. The labelling experiments were complicated by the observation that the 2-azido group of the employed nucleotide analogues have an affinity for the [4Fe-4S] cluster in the Fe protein. An additional complication is that the formed bond between protein and label is not stabile in the presence of the reductant 2-mercaptoethanol. However, a fraction of 2-azido MgADP and 2-azido MgATP specifically labels the nucleotide binding site by a stable bond. It seems reasonable to expect that a labelled peptide fragment which forms part of the nucleotide binding site of Av2 can be isolated from a trypsin digest. Determination of the amino acid sequence of the labelled peptide fragment and matching with the known amino acid sequence of Av2 should confirm the nucleotide binding site on the Fe protein as proposed by Georgiadis *et al.* [6] on basis of an X-ray diffraction study.

The autoradiographs of labelled, electrophorized Ac1 and Av1 provided no evidence that these proteins are specifically labelled on a nucleotide binding site. By gel-filtration it could be demonstrated that 2-N_3-MgADP has an affinity for a slowly dissociating site present on Ac1, but not on Av1. Coeluted 2-N_3-MgADP was found to label Ac1 with 80% efficiency. This observation proves that the azido group is in close contact with one or more amino acid residues. However, it was also found that the label is lost during electrophoresis. This may be explained by assuming that the slowly dissociating nucleotide binding site on Ac1 contains one of more cysteine residues which are labelled by 2-azido-MgADP. By analogy with the results obtained with Av2, the formed S-N bond is unstable and label is lost during electrophoresis.

6.3 *Pre-steady-state hydrolysis of MgATP by nitrogenase*

The report by Eady *et al.* [3] indicates that at 10 °C the MgATP dependent, pre-steady-state electron transfer from Kp2 to Kp1 is directly coupled to an ATPase activity of the nitrogenase complex. It was concluded that the amount of P_i released corresponds with one MgATP hydrolysed for each electron transferred to Kp1. Since the steady-state ATP:e ratio was known to be 2, the authors proposed that an additional MgATP is hydrolysed in a second reaction step. Recently, Thorneley *et al.* [7] have performed rapid-quench experiments with the nitrogenase from *K. pneumoniae* at 6 °C. At this reaction temperature no pre-steady-state P_i production could be observed. This observation was explained by proposing that at 6 °C the cleavage of MgATP on nitrogenase is reversed upon quenching in acid.

Hageman *et al.* [4] have used the rapid-quench technique to investigate the pre-steady-state ATPase activity of the nitrogenase from *A. vinelandii* at 30 °C. If it is taken into account that the authors have used a suboptimal Av2:Av1 ratio, their data are consistent with an ATP:2e ratio of 2. Cordewener *et al.* [5] reported that the dithionite reduced nitrogenase from *A. vinelandii* can hydrolyse 4 mol MgATP/mol Av2 in the pre-steady-state phase of the nitrogenase reaction. Since Av2 has two binding sites, it was proposed that the other 2 MgATP are hydrolysed on binding sites which are created in the pre-steady-state phase of the nitrogenase reaction. The same authors reported that also the dye-oxidized form of the nitrogenase complex hydrolyses 4 MgATP/mol Av2(ox) in a burst reaction.

Though never commented on in the literature, the rapid-quench data published by Hageman *et al.* [4] and Cordewener *et al.* [5] suggest that the hydrolysis of MgATP occurs after electron transfer from Av2 to Av1, thus contradicting the Lowe-Thorneley model in which electron transfer from the Fe protein to the MoFe protein is preceded by the hydrolysis of MgATP.

In Chapter 3 of this thesis it is reported that the results of Cordewener *et al.* [5] have been partly in error due to an apparatus artefact. It was discovered that the used rapid mixing apparatus caused a backflow of mixed reactants after each push. This pre-reacted reaction mixture introduced inorganic phosphate which is unrelated to the pre-steady-state ATPase activity of the nitrogenase complex. An additional artefact is that acid quenched reaction mixtures of the separate nitrogenase proteins and MgATP were found to contain orthophosphate above the background level. Repetition of the experiments with a modified rapid mixing apparatus gave no data which can be used to determine the pre-steady-state kinetics of MgATP cleavage by nitrogenase.

As a whole, great difficulties are encountered in producing consistent rapid-quench data on the pre-steady-state ATPase activity of the nitrogenase complex. This must probably be taken as compelling evidence that the rapid-quench technique is unreliable as a method for investigating the pre-steady-state ATPase activity of the nitrogenase complex.

Recently, Thorneley *et al.* [7] have used the stopped-flow microcalorimetry technique to monitor at 6 °C the MgATP dependent proton production by the nitrogenase from *K. pneumoniae*. An advantage of this technique is that the ATPase activity of the nitrogenase complex can be studied in reaction mixtures which have been unperturbed by a quenching technique. The authors concluded that MgATP hydrolysis precedes MgATP dependent electron transfer within the nitrogenase complex. It is demonstrated in Chapter 3 that pre-steady-state MgATP dependent proton production by the nitrogenase complex can also be investigated by monitoring the absorbance change of the pH indicator cresol red in a stopped-flow spectrophotometer. Like the microcalorimetry technique, this method has the advantage that MgATP hydrolysis can be observed without the need to acid-quench the reaction mixture.

The data presented in Chapter 3 place MgATP hydrolysis after electron transfer from the Fe protein to the MoFe protein. This conclusion contrasts with the data obtained with the nitrogenase from *K. pneumoniae* and the microcalorimetry technique [7]. More work is needed before this discrepancy can be understood. However, as shown in section 6.4 this inconsistency does not need to prevent the development of a reasonable model for the role of MgATP in the catalytic cycle of nitrogenase.

6.4 *Effect of reaction temperature on electron transfer*

Chapter 4 analyses the temperature dependence of the observed rate constant for MgATP dependent electron transfer. A significant observation was that at any reaction temperature electron transfer form Av2 to Av1 can be fitted to a single-exponential function. This observation proves that electron transfer is not necessarily preceded by hydrolysis of MgATP on a time scale of the same order of magnitude as reported by Thorneley *et al.* [7]. The temperature dependence of the first-order rate constant for electron transfer has been interpreted in terms of the transition-state theory and shows that electron transfer involves the formation of a highly disordered transition-state.

The analysis must be regarded as a first approximation, since more refined theories have been developed for the kinetics of electron transferring reactions. A brief introduction will be given below and may serve as a basis to consider the nitrogenase proteins as a redox-couple with kinetics of electron transfer similar to that of between other metalloproteins [8].

Redox reactions are known to proceed via either an inner-sphere mechanism or outer-sphere mechanism. The inner-sphere mechanism involves a ligand which is shared by both reactants. Electron transport involving a metalloprotein must generally

described by an outer-sphere mechanism, which implies that the electron donor and electron acceptor do not share a ligand. The kinetics of electron transfer via the outer-sphere mechanism is described by the Marcus theory [8,9] for electron transfer. The rate constant for unimolecular electron transfer is given by [8,9]:

$$k=\nu.\kappa(r).\exp(-\Delta G^{\circ\ddagger}/RT) \qquad (1)$$

In which ν is the frequency factor for nuclear motion (10^{12}-10^{14} s^{-1}). The free energy for activation is given by:

$$\Delta G^{\circ\ddagger} = (\lambda/4).(1 + \Delta G^{\circ}/\lambda)^2 \qquad (2)$$

Where ΔG° is the standard free energy change for the redox-reaction. $\lambda/4$ is the free energy change associated with the shift in nuclear conformation necessary to form the transition-state. λ can be divided in a term describing rearrangement in the reactant (inner sphere) and the solvent coordination shell (outer sphere). So that:

$$\lambda = \lambda_{in} + \lambda_{out} \qquad (3)$$

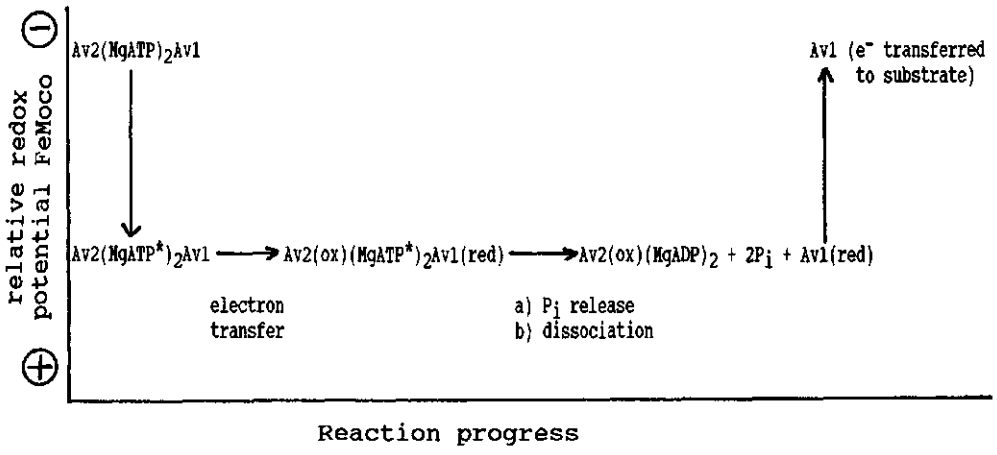
The rate for electron transfer is also dependent on the distance of the redox centres. This can be taken into account by defining a transmission coefficient, $\kappa(r)$, for electron transfer:

$$\kappa(r)=\exp(-\beta d) \qquad (4)$$

In which d is the separation between the redox-centres and β is a constant (~12 nm).

How can one hypothesize on the role of MgATP within the framework of the Marcus theory for nonadiabatic electron

transfer? It is known that both MgADP and MgATP lower the redox-potential of the Fe protein [10]: E_m(Av2) = -373 mV; E_m(Av2(MgADP)2) = -473 mV; E_m(Av2(MgATP)2) = -435 mV. The surprising situation arises that Av2(MgADP)$_2$ is a stronger reductant than Av2(MgATP)$_2$. Then why is only the Av2(MgATP)$_2$ species effective as reductant of Av1? It is known that MgATP drastically alters the conformation of the Fe protein and it might be possible that this conformation only is essential for electron transfer. However, this proposal does not explain why hydrolysis of MgATP is obligatory for catalytic activity. As demonstrated by Mortenson *et al.* [11], the hydrolysis of 5'-[γ-thio]triphosphate proceeds with inversion of the [γ-thio]phosphate group. This implies that hydrolysis of



Scheme 1. *Proposal for the pre-steady-state behaviour of MgATP in the nitrogenase reaction.* MgATP is thought to facilitate electron transfer by raising the redox potential of the FeMo-cofactor, possibly with the concomitant destabilization of the P_γ-OP_β bond of MgATP. Release of P_i is thought to occur after electron transfer from the Fe protein to the MoFe protein.

this ATP analogue occurs in a single reaction step (or an unlikely 3-step) mechanism. So, one is compelled to view nitrogenase as an ATPase whose only function is to react with water.

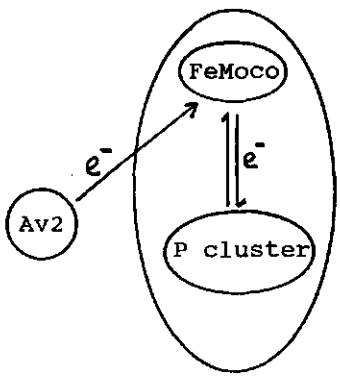
It is proposed here that, prior to electron transfer, each of 2 MgATP rearranges a water molecule from the solvation shell of the Fe protein. The hydrated MgATP remains enzyme bound at this stage, possibly with a weakened terminal phosphate group. The rearrangement in the solvation shell of the Fe protein is thought to transduce a conformational change in the MoFe protein which raises the redox potential of the super-reduced form of the FeMo-cofactor, so that electron transfer from the Fe protein to the FeMo-cofactor becomes thermodynamically favourable. Complete cleavage (*i.e.* formation of $MgADP+P_i+H^+$) might then occur after electron transfer and facilitate dissociation of the nitrogenase complex. Relaxation of the high-potential to the low-potential conformation of the dissociated MoFe protein then results in electron transfer from the FeMo-cofactor to enzyme bound substrate. See Scheme 1 for a schematization of this reaction mechanism.

6.5 *Comparison of the extent of reduction of FeMoco as judged by stopped-flow spectrophotometry and rapid-freeze EPR spectroscopy*

As demonstrated in Chapter 4, the temperature dependence of the absorbance change at 430 nm which accompanies electron transfer from the Fe protein to the MoFe protein can be explained by assuming reversible electron transfer. However, in Chapter 5 experiments are described in which the redox-state of the nitrogenase proteins have been investigated by rapid-freeze EPR spectroscopy in combination with stopped-flow spectroscopy. It was found that NaCl diminishes $\Delta A(430)$. Addition of 500 mM NaCl lowered $\Delta A(430)$ to approximately 30% of the maximal value.

Nevertheless, it was demonstrated in a rapid-freeze EPR experiment that 90% of the FeMo cofactor is reduced in the presence of 500 mM NaCl. Also, rapid-freeze EPR spectroscopy shows that 80% of the MoFe cofactor is reduced at 6 °C, whereas stopped-flow spectroscopy suggests that 50% of the cofactor is reduced. It can thus not be confidently assumed that the diminished value for ΔA(430) induced by a low reaction temperature or the addition of NaCl must be ascribed to a redox-equilibrium between the Fe protein and MoFe protein of the nitrogenase complex.

As noted in the discussion of Chapter 5, there are no solid experimental data available which explain the discrepancy between the stopped-flow spectrophotometry and rapid-freeze EPR experiments. Though the data indicate that electron transfer between the nitrogenase proteins must be considered as irreversible. A speculative model which explains the observations of Chapter 5 is outlined in Scheme 2. It is suggested that at room temperature and in the absence of NaCl the FeMo-cofactor accepts two electrons in the pre-steady-state phase of the nitrogenase reaction. One electron is supplied by the Fe protein in an irreversible reaction and the second



Electron transfer:

1) Fe protein ---> FeMoco
ΔA(430) = 0 mM^{-1} Mo·cm^{-1}

2) P cluster ---> FeMoco
ΔA(430) = 5.5 mM^{-1} Mo·cm^{-1}

Scheme 2. *Proposal for the effect of low reaction temperatures and NaCl on pre-steady-state electron transfer within the nitrogenase complex.*

electron synchronously in a reversible reaction by a P cluster. Electron transfer from the Fe protein to the FeMo-cofactor is assumed to occur without an absorbance change. The absorbance change as monitored by stopped-flow spectrophotometry is thought to originate from oxidation of the P cluster.

At a low reaction temperature or in the presence of NaCl the redox-equilibrium between the P cluster and FeMoco is shifted in favour of the P cluster. Within the framework of Scheme 2 this results in a decrease of the monitored absorbance change and a reduction of the FeMo-cofactor by less than two electrons.

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SAMENVATTING

Eén van de belangrijkste moleculen in de plantenwereld is ammoniak (NH_3). Uitgaande van ammoniak synthetiseert de plant biomoleculen zoals eiwitten en nucleïnezuren. Planten zelf zijn niet in staat moleculair stikstof (N_2) te reduceren tot ammoniak. De biologische reductie van N_2 tot NH_3 wordt bewerkstelligd door diazotrope bacteriën. Deze bacteriën bevatten het enzymcomplex nitrogenase. Nitrogenase bestaat uit twee zuurstofgevoelige metalloproteïnen die afzonderlijk geïsoleerd kunnen worden.

De grootste van deze metalloproteïnen is een $\alpha_2\beta_2$-tetrameer. Elke αß-unit functioneert als een onafhankelijke katalytische eenheid. De feitelijke reductie van N_2 vindt plaats op een ijzer-molybdeen cofactor (FeMoco). De voor de reductie benodigde elektronen worden *in vivo* geleverd door een ferredoxine of flavodoxine. Het MoFe-eiwit is niet in staat deze elektronen rechtstreeks op te nemen. In de katalytische cyclus van nitrogenase staat het ferredoxine of flavodoxine een elektron af aan de tweede component van het nitrogenase complex. Deze component is een Fe-eiwit, welk het elektron vervolgens aan het MoFe-eiwit doorgeeft.

Reductie van een metalloproteïn door een ander metalloproteïn is een veel voorkomende reactie in de biochemie. Het bijzondere van nitrogenase is dat elektronoverdracht slechts in aanwezigheid van magnesium-adenosinetrifosfaat (MgATP) optreedt. Experimenten verricht onder steady-state condities hebben laten zien dat per elektron dat naar een substraat wordt getransporteerd, er twee MgATP moleculen worden gehydrolyseerd. Geruime tijd bestond er onzekerheid over de vraag waar in de katalytische cyclus MgATP wordt gehydrolyseerd. In 1978 publiceerde de Brighton groep een experiment waaruit werd geconcludeerd dat de hydrolyse van MgATP en elektrontransport

van het Fe-eiwit naar het MoFe-eiwit direct gekoppeld zijn. De gevonden ratio mol MgATP gehydrolyseerd/mol elektron overgedragen was gelijk aan 1. De auteurs interpreteerden dit resultaat als een aanwijzing dat elders in de katalytische cyclus ook MgATP wordt gehydrolyseerd.

De conclusie van de Brighton groep dat elektrontransport van het Fe-eiwit naar het MoFe-eiwit en de hydrolyse van MgATP gekoppeld zijn, werd algemeen aanvaard. Echter, een punt van discussie bleef de ratio mol MgATP gehydrolyseerd/mol elektron overgedragen. In 1980 verscheen een publikatie uit de groep van Burris waarin werd geconcludeerd dat deze ratio gelijk is aan 2, hetgeen impliceert dat de hydrolyse van MgATP alleen plaats vindt bij elektronoverdracht van het Fe-eiwit naar het MoFe-eiwit. Echter, in 1987 verscheen een publikatie van de vakgroep Biochemie uit Wageningen waarin werd gerapporteerd dat er 4 moleculen MgATP gehydrolyseerd kunnen worden per mol Fe-eiwit. Het Fe-eiwit heeft twee bindingsplaatsen voor MgADP of MgATP. Deze stoichiometrie impliceert dat er niet meer dan twee MgATP moleculen gehydrolyseerd kunnen worden per turnover van het Fe-eiwit. Teneinde een ratio van 4 te kunnen verklaren, moest worden aangenomen dat twee extra bindingsplaatsen ontstaan wanneer het Fe-eiwit en MoFe-eiwit associëren.

Het doel van dit proefschrift was duidelijkheid te krijgen in de rol die MgATP vervult in de pre-steady-state ATPase activiteit van nitrogenase. In hoofdstuk 2 wordt een onderzoek beschreven waarin 2-azido-analogen van MgAMP, MgADP, en MgATP worden gebruikt om de nucleotide bindingsplaatsen binnen het nitrogenase complex te karakteriseren. Met deze experimenten zijn geen aanwijzingen verkregen dat in het nitrogenase complex nucleotide bindingsplaatsen bestaan naast die op het Fe-eiwit. Wel is duidelijk geworden dat Av2(ox) twee verschillende soorten bindingsplaatsen heeft die van belang zijn voor labellings experimenten met azido-nucleotiden. Een van de bindingsplaatsen heeft een affiniteit voor de azidogroep en wordt geïdentificeerd

als het [4Fe-4S] cluster. De tweede bindingsplaats is specifiek voor MgADP en MgATP. Een deel van de gevormde bindingen wordt gereduceerd door 2-mercaptoethanol, hetgeen resulteert in verlies van label door het gemodificeerde eiwit.

In hoofdstuk 3 wordt aangetoond dat de door de Wageningse onderzoeksgroep gerapporteerde burst reactie van 4 moleculen MgATP gehydrolyseerd/mol Av2, veroorzaakt is door een apparaat artefact. Een herhaling van de experimenten met verbeterde rapid quench apparatuur gaf geen bevestiging van het algemeen aanvaard mechanisme waarin elektronoverdracht vooraf wordt gegaan door een volledige hydrolyse van MgATP. Er wordt ook op gewezen dat de gepubliceerde rapid-quench experimenten tegenstrijdige antwoorden geven op de vraag of elektronoverdracht voor dan wel na hydrolyse van MgATP plaats vindt. Tevens wordt beschreven hoe protonproduktie in de pre-steady-state fase van de nitrogenase reactie gevolgd kan worden met een stopped-flow spectrofotometrie techniek. Bij deze techniek wordt de kleursverandering van de pH-indicator cresol rood gebruikt om protonproduktie waar te nemen. De waargenomen snelheid van protonproduktie ligt lager dan die van elektronoverdracht.

In hoofdstuk 4 wordt de temperatuursafhankelijkheid van de MgATP-afhankelijke elektronoverdracht van het Fe-eiwit naar het MoFe-eiwit bekeken. De amplitude van het stopped-flow signaal is afhankelijk van de reactietemperatuur. Deze waarneming wordt verklaard door aan te nemen dat elektronoverdracht van het Fe-eiwit naar het MoFe-eiwit reversibel is. De snelheid van elektronoverdracht vertoont een interessante temperatuurs-afhankelijkheid. De resultaten zijn geanalyseerd in het kader van de transition-state theorie. Dit laat zien dat het geactiveerde complex gekarakteriseerd wordt door een uitzonderlijk hoge activeringsentropie. Deze waarneming is een aanwijzing dat elektronoverdracht gepaard gaat met een grote conformatieverandering binnen het nitrogenase complex.

In hoofdstuk 5 wordt bij 23 °C het effect bestudeerd van

een hoge NaCl-concentratie op de extinctieverandering bij 430 nm, die gepaard gaat met elektrontransport van het Fe-eiwit naar het MoFe-eiwit. Net zoals een lage reactietemperatuur verlaagt NaCl deze extinctieverandering. Rapid-freeze EPR experimenten geven niet aan dat deze waarnemingen zonder meer verklaard mogen worden door een reversibele elektronoverdracht tussen beide nitrogenase eiwitten aan te nemen.

In hoofdstuk 6 van dit proefschrift is een algemene discussie van de resultaten gegeven. Er wordt geconcludeerd dat er in dit proefschrift geen eenduidige aanwijzingen zijn verkregen die aantonen dat volledige hydrolyse van MgATP noodzakelijk is voor elektronoverdracht tussen de nitrogenase eiwitten. Daarom wordt een alternatief gegeven voor modellen die in de literatuur zijn verschenen.

Curriculum vitae

Richardus Engelbertus Mensink werd geboren op 30 mei 1958. In 1974 behaalde hij het diploma Mavo IV aan de Professor Kohnstamm Mavo te Velp (Gld). Na het doorlopen van het voorbereidend jaar voor het HBO-A onderwijs aan de OLAN (Opleiding Laboratorium Personeel Arnhem Nijmegen) werd in 1975 aangevangen met een twee-jarige opleiding voor chemisch analist. Het laatste schooljaar omhelsde een stage periode op het Kernreactor Laboratorium van de KEMA te Oosterbeek.

In 1977 schreef de auteur van dit proefschrift zich in aan de faculteit voor Wiskunde & Natuurwetenschappen van de Katholieke Universiteit te Nijmegen voor de opleiding MO-A natuur/scheikunde. In 1978 werd hem een Colloquium Doctum verleend, waarna hij zijn studie voortzette volgens het kandidaatsrooster Scheikunde (S2). Het kandidaatsexamen werd in 1981 afgelegd. Het doctoraalexamen werd in 1984 afgelegd met als hoofdvak Biofysische Chemie (Dr. S.H. de Bruin), en de bijvakken Farmacochemie (Prof. J. van Rossum) en Molecuulspectroscopie (Dr. C. Keysers).

Van augustus 1984 tot en met december 1985 heeft hij als Erkend Gewetensbezwaarde onder leiding van professor J. van Rossum op het Laboratorium voor Dopingcontrole onderzoek gedaan naar de detectie van metabolieten van anabole steroïden in menselijke urine.

Van januari 1986 tot januari 1990 was hij als promovendus verbonden aan het Laboratorium voor Biochemie van de Landbouwuniversiteit te Wageningen. De werkzaamheden verricht op dit laboratorium hebben geresulteerd in dit proefschrift.