ELECTRON TRANSPORT THROUGH THE NITROGENASE ENZYME COMPLEX OF AZOTOBACTER VINELANDII

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Proefschrift
ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. C.C. Oosterlee,
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ISN : 234131

STELLINGEN

1.De conclusie van Fuller en Verma dat de soya leghaemoglobine genen reeds drie dagen na inoculatie tot expressie komen, moet in twijfel getrokken worden.

Fuller,F. en Verma,D.P.S. (1984) Plant Molecular Biology 3,21-28.

2.Gezien het feit dat met flavodoxine als electronendonor een veel hogere specifieke activiteit voor het Fe-protein te bereiken is in vergelijking met dithioniet als electronendonor, is het in deze tijd van superlatieven, noodzakelijk de tot nu toe bestaande en algemeen gebruikte standaard activiteitsmeting te wijzigen.

Hageman,R.V. en Burris,R.H. (1978) Biochemistry 17,4117-4124.
Scherings,G.,Haaker,H. en Veeger,C. (1977)
Eur.J.Biochem.77,621-630.
Dit proefschrift.

3.De suggestie van Orme-Johnson en Davis dat de binding van MgATP een verlaging van de intrinsieke redoxpotentiaal van het Fe-eiwit veroorzaakt, kan niet worden gedaan zonder de bindingsconstanten van MgATP aan het geoxideerde en gereduceerde Fe-eiwit erin te betrekken.

Orme-Johnson,W.H. en Davis,L.C.(1977) In:Iron-Sulfur Proteins III, Lovenberg,W.,ed. Academic Press, New York. p.38.

4. Het gebruik van de term "energieproductie" als jargon door biochemici is in strijd met de eerste hoofdwet van de thermodynamica en zou consequent veranderd moeten worden in "energieomzetting".

5.Correctie voor het aanwezige inactieve Fe-protein in een preparaat wordt soms ten onrechte toegepast en kan leiden tot "wishful thinking".

Ljones,T. en Burris,R.H.(1978)Biochem.Biophys.Res.Comm.80,22-25.
Huynh,B.H.,Henzl,M.T.,Christner,J.A.,Zimmerman,R.,Orme-Johnson,W.H en Munck,E. (1980) Biochim.Biophys.Acta 623,124-138.

6.Gezien het effect van de groeiomstandigheden op sommige eigenschappen van het te isoleren Fe-protein van nitrogenase, kan men nu niet meer volstaan met uitsluitend het vermelden van de samenstelling van het groeimedium van de bacterie in een publicatie over de eigenschappen van het nitrogenase enzymcomplex.

Dit proefschrift.

7.Het is hoogst wenselijk, dat de parameter voor de hydrofobiciteit voor biokatalytische reacties in multifase systemen gestandariseerd wordt.

Hilhorst,R.,Spruyt,R.,Laane,C. en Veeger,C.(1984) Eur.J.Biochem.144,459-466.
Brink,L.E.S. en Tramper,J.(1985) Biotechnol.Bioeng.27,1258-1269.

8.Met een redelijke mate van waarschijnlijkheid kan gesteld worden, dat de abnormale divergentie in hyperfijn structuur van EPR poeder-spectra rond 1990 opnieuw ontdekt zal worden.

Bleaney,B.(1951) Phil.Mag.42,441-458.
Nieman,R. and Kivelson,D.(1961)J.Chem.Phys.35,156-161.
Chen,I.,Abkowitz,M. and Sharp,J.M.(1969) J.Chem.Phys. 50,2237-2244.
Lee,S.,Ames,D.P. and Putnam,J.M.(1982) J.Magn.Reson.49,312-321.

9.Het bestaan van een ijzer-chlorose signaal tussen de spruit en de wortels, kan op grond van de huidige literatuur gegevens nog niet uitgesloten worden.

Bienfait,H.F. (1985) J.Bioenerg.Biomembr.17,73-83.

10.De Michelingids zou aan die restaurants die de nouvelle cuisine nastreven, behalve een kwaliteits- ook een kwantiteitsaanduiding voor de maaltijd moeten geven.

A.Braaksma
Electrontansport through the nitrogenase enzyme complex of Azotobacter vinelandii.
Wageningen,november 1985.

Zwavels meten,
IJzers meten,
Zuurstof slaat hier snel z'n slag,
Váak werd toen de zucht geslaakt,
Als'k dit nog één keer meten mag.

Aan
Els,
Pieter-Jelle en
Laurens

ISN= 234131.03

Voorwoord

Het tot stand brengen van een proefschrift wordt vaak als een grote prestatie gezien. Mocht dit zo zijn, dan is dit zelden of nooit geheel aan de verdienste van één persoon toe te schrijven. Weliswaar was ik de persoon die moest volhouden tot het klaar was, maar dat kan alleen als je daartoe in staat wordt gesteld.

Allereerst dank ik daarvoor mijn moeder die me in staat stelde biologie te studeren.

Een speciaal woord van dank voor mijn co-promotor Huub Haaker, die mij met een niet aflatende energie over de dode punten heen hielp. Hij deed dit altijd op een warme, menselijke wijze die ik zeer op prijs heb gesteld en waar ik grote bewondering voor heb.

De vaste bewoners van lab. III, de beide Jannen en Hans, dank ik voor de fijne samenwerking en sfeer die de resultaten zeker ten goede kwamen.

Verder dank ik mijn promotor professor Veeger voor zijn begeleiding bij de tot standkoming van dit proefschrift.

I am also indebted to Bob Eady who made this thesis more readible, not only by correcting the text into proper English if necessary, but also by making contributions in content.

Voor het niet geringe typewerk dank ik Jenny Toppenberg en voor het tekenwerk Martin Bouwmans.

Verder dank ik iedereen op het laboratorium voor de fijne tijd die ik daar heb gehad.

Tenslotte mag niet onvermeld blijven dat dit proefschrift mogelijk werd gemaakt door de financiële steun van de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek (Z.W.O.).

Contents

List of abbreviations

A_{430}	Absorbance at 430 nm.
ADP	Adenosine 5'-diphosphate
ATP	Adenosine 5'-triphosphate
ATPase	ATP phosphohydrolase (E.C. 3.6.1.3)
BPS	Bathophenantroline disulphonate
DEAE	Diethylaminoethyl
Deazaflavine sulphonic acid	10-methyl-5-deazaisoalloxazine-N-3-propyl-sulphonic acid
DNA	Deoxyribonucleic acid
ENDOR	Electron nuclear double resonance
EPR	Electron paramagnetic resonance
Hepes	4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid
MV	Methyl viologen
Pi	Inorganic Phosphate
PIXES	Proton induced X-ray emission spectroscopy
Tricine	N-[2-hydroxy-1,1-bis(hydroxy-methyl) ethyl]glycine

The MoFe and Fe-proteins of the nitrogenase of Azotobacter vinelandii, Clostridium Pasteurianum and Klebsiella pneumoniae are referred to as Av_1 and Av_2, Cp_1 and Cp_2 and Kp_1 and Kp_2 respectively.

INTRODUCTION

Nitrogenase in the evolution

Living organisms are composed primarily of four elements: carbon, oxygen, hydrogen and nitrogen. These four elements combine in various ways to make up the four main classes of organic compounds as carbohydrates, fats, proteins and nucleic acids. The reason why life consists of these four elements must be searched at the origin of life. The composition of the early earth atmosphere was probably methane, ammonia, dinitrogen, dihydrogen and water vapour [1-3]. It has been shown in experiments where primitive atmospheric conditions were imitated that in these reducing surroundings organic molecules can be formed spontaneously in such a variety that it seems as if whole organisms have been desintegrated into their building blocks [2,4]. Even in these early stages one can speak of selection in evolutionary terms. For instance conditions changed as oxygen accumulated up to a concentration of 1% probably by photochemical reactions by sunlight [3,5]. By a gradually changing environment the tendency to form aggregates was favoured. Even abiotic cell-like structures could be formed. This chemical evolution, as it is called, is thought to have led to self-replicating organic structures, or in other words: to life.

Some 3 billion years ago there was probably a variety of life, but without fossilizable structures. Anaerobic organisms using inorganic comounds as energy source (SO_4^{2-}), were probably the main forms of life on earth at that time. The next possible stage in the evolution process might have been the development of photosynthetic bacteria. They could use solar radiation as an energy source and metabolise both organic and inorganic compounds. The photosynthetic bacteria thus need not compete for the depleting supply of organic molecules as a source of energy [6]. Almost all photosynthetic bacteria are able to use N_2 as a source of nitrogen, this process is called nitrogen fixation and it is catalyzed by the enzyme nitrogenase [1]. But it might well be that the nitrogenase enzyme in the beginnning did not have a function in N_2 fixation but functioned to discharge electrons in the form of dihydrogen like Rhodospirillum rubrum

today still does when it is growing on an organic compound. One of the oldest fossils are cyanobacteria [6], many representatives fix dinitrogen today. This might indicate that nitrogen fixation appeared early in evolution. Recently, nitrogen fixation has been discovered to occur in archaebacteria [7]. So the question arises, as to whether nitrogen fixation is so ancient a property that it originated before divergence of archaebacteria and eubacteria or did diazotrophy originate separately in the two kingdoms? So it might be that the nitrogen fixing process is very old. The structure of the enzyme (nitrogenase) that converts dinitrogen into ammonia isolated from many eubacteria, is remarkably conserved. Not only the isolated nitrogenase proteins are very similar but DNA hybridisation studies indicate that the structural nif genes are highly conserved in all Diazotrophs investigated [8].

Significance of biological nitrogen fixation

The nitrogenase enzyme complex is the only biological input system of dinitrogen into the N-cycle. To keep the N-content of a soil constant the loss of fixed nitrogen as dinitrogen by denitrification must be compensated for by an input from chemical and biological nitrogen fixation. In many areas on earth the availability of fixed nitrogen is rate-limiting for plant growth [9].
Today we have the possiblity of the industrial nitrogen fixation according to the Haber-Bosch process, but it is only applied to agriculturally important land [9]. Chemical fertilizers have some disadvantages. For instance, it washes out rapidly which leads to pollution of nearby water. The cost of producing chemical fertilizers, storage of the supplies, transport etc. is within the financial reach of rich countries, but not of that of most developing countries. Such undesirable side effects are not, or are only to a small extent, applicable to biological nitrogen fixation.

This thesis is a study of the nitrogenase enzyme of the aerobic bacterium Azotobacter vinelandii, chosen mainly because it can be grown easily and nitrogenase can be isolated in pure form. Since nitrogenases have very similar properties, irrespective of the source from which they are isolated, most conclusions can be extended to other organisms [10,11].

Structure and function of nitrogenase

The enzyme Nitrogenase

Nitrogenase, officially called dinitrogen oxidoreductase, ATP hydrolysing (E.C. 1.18.2.1), consists of two distinct components, both required for activity [9,10,11]. During the isolation of the enzyme, these two components are usually separated.

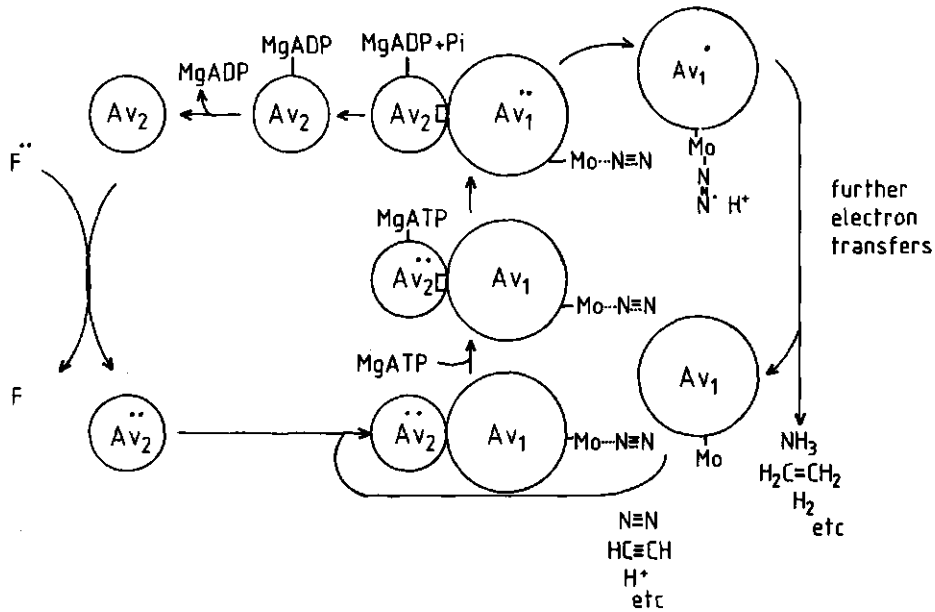
The largest component is a tetrameric protein with a molecular mass of 220-245 kDa for the native protein. Three names are in common use: component I; MoFe-protein; or an abbreviation in which the first two letters of the bipartite latin name of the organism from which it is isolated, followed by the number '1'. To illustrate this, Av_1 is nitrogenase component I isolated from Azotobacter vinelandii [10,11].

Component I contains two molybdenum atoms, 28-32 iron atoms and at least 28 acid labile sulfur atoms per molecule. It is suggested that the catalytic site is situated on this protein [10,11] which contains several types of redox centres. Approximately half the Fe is present in a Fe and Mo containing cofactor (FeMoCo) and the remainder in so-called 'P' clusters which are suggested to be [4Fe-4S] clusters and a minority in the 'S' clusters [12]. FeMoCo is supposted to be the substrate binding site (13). The necessary electrons for this process are probably stored and tunneled through the [4Fe-4S] clusters, to the FeMo, where dinitrogen is reduced to ammonia by electron and proton addition, a process in which dihydrogen is also evolved [20,21]. The smaller nitrogenase component seems simpler in structure, yet there are some intriguing questions about its structure and mode of action. In the literature this protein is referred to as component II; Fe-protein; or abbreviated (Av_2). Fe-protein is a dimeric protein [11], consisting of two identical subunits, and the native protein has a molecular mass of about 63 kDa. The main function of component II is to transfer electrons to component I in an ATP dependent reaction.

Nitrogenase catalyses the reaction:

$$N_2 + \geqslant 16\ MgATP + 8e + 8\ H^+ \rightarrow 2\ NH_3 + H_2 + \geqslant 16\ MgADP + \geqslant 16\ Pi.$$

The nitrogenase reaction is visualised in figure 1 (after Postgate [9] slightly adapted).



In Fig. 1, F is the electron donor. In vitro dithionite is the most widely used donor. The electron donor in vivo is not known with certainty for every organism. In Clostridium pasteurianum it is probably a ferredoxin [14]. However, in K.pneumoniae it has been shown by genetic experiments that the product of the nif F gene is necessary for in vivo activity of nitrogenase. This gene codes for a flavodoxin and is an integral part of the nif gene cluster [15,16]. In Azotobacter circumstantial evidence points in the direction of flavodoxin as a possible donor in vivo [23,24]. Reduction, ATP binding to component II and complex formation are thought to occur at random. It has been reported earlier that on component II there are two binding sites for MgATP [17-21]. Recent results indictate that

these two binding sites interact with negative cooperativity [22]. Oxidized Av_2 binds two molecules MgATP per molecule Av_2 with equal binding constants [23].

Once the complex has been formed rapid electron transfer takes place with the concommittant hydrolysis of MgATP. Since oxidized component II cannot be reduced when bound to component I a dissociation of the proteins must occur before component II can be rereduced. The rate of dissociation is thought to be the slowest step in nitrogenase catalysis [10]. The whole cycle has to be repeated until enough electrons are transferred to component I to allow substrate reduction.

Iron-Sulphur clusters in Nitrogenase

The electrons participating in the oxidation-reduction processes in nitrogenase are stored in iron-sulphur clusters. Today several types of clusters are known.

The main characteristic of these clusters is the alternation of iron and sulphur atoms and the fact that they are incorporated in the proteins through a covalent binding between cysteine residues and the iron atoms. At the moment the Rieske iron-sulphur protein seems to be the only known exception: here the cluster is bound to two cysteine residues and two non-cysteine residues [24].

In figure 2 some known structures of iron/sulphur clusters are shown.

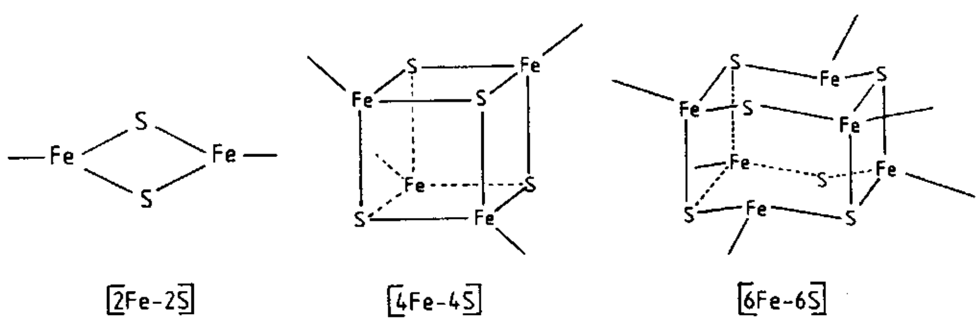


Figure 2. Structures of some iron/sulphur clusters.

Iron-suphur clusters are always paramagnetic in one of their redox states. This paramagnetism can be monitored by Electron Spin Resonance spectroscopy (EPR) and thus the oxidation/reduction state of the protein determined. The direction of electron transfer through nitrogenase as shown in fig. 1 has been established primarily through EPR experiments. As isolated in the presence of dithionite each protein has a characteristic EPR signal and during turnover the EPR signal of each protein is reduced in intensity. In combination with other experiments, it has been shown that during turnover Fe-protein is partly oxidized and MoFe-protein is in a more reduced state. It is clear that EPR spectroscopy is a powerful technique in studying electron transport through nitrogenase but one of the biggest problems in interpreting experimental data for nitrogenase is the uncertainty as to which extent the preparations are homogeneous with respect to the metal clusters. The composition of the metal clusters is one of the main subjects of this thesis.

Scope of this thesis:

This thesis deals with some properties of the MoFe-protein of nitrogenase from A.vinelandii (Av_1) and of Fe-protein of nitrogenase (Av_2). In the different chapters attention is focussed upon specific aspects.
In Chapter II the redox potential necessary for nitrogenase activity in vitro is determined. In this chapter we also show that Av_2 can behave as a two-electron donor instead of the expected one electron donor.

In Chapter III we show that enzymatically oxidized or dye-oxidized Av_2 can take up two electrons per molecule.

In Chapter IV we show that the iron-sulphur content of Av_2 is variable and can exceed 4 iron and 4 sulphur atoms per molecule of Av_2. This is not consistent with the generally accepted view that the iron-sulphur cluster in Av_2 is a ferredoxin type of [4Fe-4S] cluster.

In Chapter V it is shown that the iron-sulphur content of isolated Av_2 is dependent upon the growth conditions of the cells.

Chapter VI deals with quantitative Mössbauer spectroscopy of Av_1. The outcome of these studies raises questions about the possible structure and function of the clusters in this protein.

In Chapter VII the results described in this thesis are discussed in relation to the present knowledge of structure and functioning of nitrogenase.

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Eur. J. Biochem. *121*, 483 – 491 (1982)

CHAPTER II.

The Effect of the Redox Potential on the Activity of the Nitrogenase and on the Fe-Protein of *Azotobacter vinelandii*

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The redox potential dependence on the nitrogenase reaction catalyzed by different nitrogenase complexes from *Azotobacter vinelandii* has been studied by two methods. The redox potential was set with the redox couple $SO_2^{\cdot-}/SO_3^{2-}$ and the effect on the nitrogenase activity was determined. The oxidation of photochemically reduced low-potential electron carriers by nitrogenase was followed spectrophotometrically. Simultaneously the nitrogenase activity was estimated by the production of hydrogen. It was found that when component II of the nitrogenase is in redox equilibrium with the electron donor, the nitrogenase activity is maximum and independent of the redox potential up to -440 mV. At higher potentials the nitrogenase activity declines and no significant activity was detectable at potentials above -350 mV.

The effect of the redox potential on the oxidation reduction state of the 4Fe-4S cluster of *Azotobacter* nitrogenase component II was studied by electron paramagnetic resonance spectroscopy. Without adenine nucleotides present, component II of the nitrogenase shows a midpoint potential of -393 mV and the oxidation reduction reaction is characterized by the transfer of two electrons per redox step. In the presence of MgATP the midpoint potential of component II of the nitrogenase undergoes a negative shift of 42 mV. The curve fitting to the experimental points is also characteristic of a two-electron redox step. In contrast, it is found that in the presence of MgADP, the curve fitting to the experimental points is characterized by a one-electron step with a midpoint potential of -473 mV. It is demonstrated that the nitrogenase activity correlates with the oxidation reduction state of component II of the nitrogenase without adenine nucleotides bound. The implications of this observation, indicating a sequential reaction of the reduced Fe protein with the Mo-Fe protein, for the present kinetic models of nitrogenase will be discussed.

Nitrogenase is the enzyme system (reduced carrier: N_2 oxidoreductase) that consists of two proteins and converts N_2, ATP-dependently, into NH_3. The nitrogenase system is composed of two dissociating protein components. One, called the Fe protein, contains four iron and four acid-labile sulphur atoms; the other, the Mo-Fe protein, contains two molybdenum, 28 – 32 iron, and about 28 acid-labile sulphur atoms. The properties of nitrogenases from bacterial sources were recently reviewed by Mortenson and Thorneley [1]. The nitrogenase reaction needs a low-potential electron donor and at least two molecules of MgATP are hydrolyzed to MgADP and P_i per electron transferred to the substrates. Electrons are donated to the Fe protein and pass to the Mo-Fe protein, ATP-dependently [1,2]. The Mo-Fe protein acts as a storage sink for electrons and passes the electrons in multiples of two to the substrates [1,2]. The electron donors *in vivo* are thought to be either a ferredoxin or a flavodoxin [3,4]. The potential electron carriers for the nitrogenase have midpoint potentials ranging from -495 mV for *Azotobacter vinelandii* flavodoxin up to -340 mV for ferredoxin III from *Rhodospirillum rubrum* [3,4]. Little information is available about the actual redox potential necessary for dinitrogen fixation. There are a few reports in the literature about the dependence of the rate of the nitrogenase-catalyzed reaction upon the redox potential of the electron donor. Evans and Albrecht [5] reported that for a partly purified nitrogenase complex of *Chromatium vinosum* an applied potential of -465 mV at pH 8.0 is required for half-maximum rates of acetylene reduction. Watt and Bulen [6] reported for the nitrogenase complex of *A. vinelandii* that at an applied voltage of -460 mV the nitrogenase complex exhibits half of its maximal H_2 production capacity. Finally Scherings et al. [7] showed that the nitrogenase complex from *A. vinelandii* did not oxidize photochemically-reduced *A. vinelandii* flavodoxin at redox potentials higher than -460 mV.

These results are not in accordance with the observations of Zumft et al. [8]. The observed for the Fe protein of *Clostridium pasteurianum* a midpoint potential of -294 mV at pH 7.5. When MgADP or MgATP was bound to the protein the midpoint potential shifted to -400 mV. If we assume similarities between the Fe protein from *Clostridium* and *Azotobacter*, then redox potentials around -460 mV must be low enough to keep the Fe-protein · MgATP complex in the reduced state and allow nitrogenase proteins to have maximum activities. To solve this discrepancy and to obtain more information on the reducing power necessary for dinitrogen fixation, we studied the effect of the redox potential on the nitrogenase activity and on the oxidation-reduction potential of the Fe protein with or without adenine nucleotides bound. To exclude specific effects of the electron donor

Abbreviations. The Mo-Fe and Fe proteins of the nitrogenases of *Azotobacter vinelandii*, *Azotobacter chroococcum*, *Clostridium pasteurianum* and *Klebsiella pneumoniae* are referred to as Av_1 and Av_2, Ac_1 and Ac_2, Cp_1 and Cp_2 and Kp_1 and Kp_2, respectively; EPR, electron paramagnetic resonance; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; deazaflavine sulphonic acid, 10-methyl-5-deazaisoalloxazine-*N*-3-propyl-sulphonic acid; G, gauss.

on the Fe protein, we used different electron donors. The implications of these results with respect to the model for nitrogenase-catalyzed electron transfer will be discussed.

MATERIALS AND METHODS

Growth Conditions and Enzyme Preparation

Azotobacter vinelandii ATCC 478 was grown in a batch culture of 2500 l, harvested during the logarithmic phase and stored at −70 °C [9]. Routinely approximately 250 g wet weight of cells were disrupted with a Manton Gaulin homogenizer type 15 M (Gaulin Corporation, Everett, MA, USA). The nitrogenase proteins were purified by the method of Yates and Planqué [10], with an additional purification step for the Mo-Fe protein by an $MgCl_2$ precipitation analogous to the $MgCl_2$ precipitation described for the three-component nitrogenase complex [11]. The nitrogenase components were freed of dithionite by passing the proteins over a Sephadex G-25 column equilibrated with 25 mM Hepes/KOH, 40 mM α-D-glucose, 0.5 mg glucose oxidase/ml, final pH = 7.4. *Azotobacter* flavodoxin was purified as described by Hinkson and Bulen [12]. *Megasphera elsdenii* flavodoxin as described by Mayhew and Massey [13]. The three-component nitrogenase complex was purified according to Bulen and LeCompte up to the second $MgCl_2$ precipitation [11].

Analytical Methods

Standard nitrogenase activity assays were run as described earlier [7] in a mixture containing 50 mM Hepes/KOH, 10 mM ATP, 15 mM $MgCl_2$, 10 mM creatine phosphate, 40 mM α-D-glucose, 1 mg/glucose oxidase/ml, 0.5 mg creatine kinase/ml, 1 mg bovine serum albumin/ml, final pH 7.0. Argon was the usual gas phase. Assay mixtures were made anaerobic by evacuating and filling with argon five times. Dithionite was added and, when acetylene reduction was measured, acetylene purified by storage for at least 24 h above a Fieser solution was added to a final concentration of 20%. After a preincubation at 30 °C the reactions were started by the addition of nitrogenase components with gas-tight syringes. Photoreduction of electron carriers was carried out in the presence of 50 mM Tricine buffer and deazaflavine-sulphonic acid, added to the standard assay mixture as described earlier [7].

Production of H_2 was monitored continuously by an amperometric method [14]. The H_2 electrode was similar in design to Clark-type O_2 electrode and the reaction chamber had a volume of 1.6 ml. The electrode was standardized by the addition of aliquots of water saturated with H_2 at 30 °C. In some experiments the whole reaction chamber was built in an Aminco DW 2 spectrophotometer connected with a Midan T microprocessor. The effective light pathway was calibrated with a dichloroindophenol solution, at the same wavelength settings as used for monitoring the concentration of electron carriers. The electron carriers were photoreduced by side illumination with a short white light pulse (10–20 s) [7]. Nitrogenase was allowed to oxidize the reduced electron carriers. The reaction was plotted by the spectrophotometer and the data simultaneously stored in the analyzer. The first-order derivative of the data was computed and plotted on the same sheet as the reaction had been plotted. From the progress curve of carrier oxidation at each point the redox potential was calculated by means of the Nernst equation. The activity of the nitrogenase was calculated from the first derivative. The calculated activities of the spectrophotometric nitrogenase assay were always in good agreement with the values for H_2 production polarographically obtained. The results of the electron carrier oxidation curves were fitted with the linear regression method to the following equation:

$$E_h = E_m + \frac{RT}{nF} \ln \frac{100 - A}{A},$$

where E_m = the redox potential when the nitrogenase activity is 50%, E_h = the redox potential observed and A = percentage of nitrogenase activity. The E_m and n values were calculated. It was found in the experiments with *M. elsdenii* flavodoxin as electron donor, that nitrogenase activities below 20% did not follow the sigmoidal curve but follow a straight line (see Fig. 2). These activity values were not taken into account for the calculation of the E_m and n values. The results of the experiments with *A. vinelandii* flavodoxin as electron donor could not be described by this method.

Nitrogenase components used had throughout specific activities around 2000 nmol C_2H_4 formed min^{-1} (mg Fe protein)$^{-1}$ and 2500 nmol C_2H_4 formed min^{-1} (mg Mo-Fe protein)$^{-1}$, when measured under standard assay conditions at pH 7.4 and optimum levels of the complementary protein. For the calculation of nitrogenase protein concentrations we used for the Mo-Fe protein M_r = 220000, a value estimated from sedimentation-equilibrium studies taking the partial specific volume of 0.73 ml g^{-1} [15]; M_r = 60000 was used for the Fe protein.

In a large series of isolations we found that the maximal specific activity obtainable for the Mo-Fe protein is 3000 nmol C_2H_4 formed min^{-1} (mg Mo-Fe protein)$^{-1}$ and 2500 nmol C_2H_4 formed min^{-1} (mg Fe protein)$^{-1}$. These values were used to calculate the amount of active protein present in each experiment.

The concentrations of the electron carriers were estimated from the absorption coefficients: oxidized *M. elsdenii* flavodoxin, 10200 M^{-1} cm^{-1} at 445 nm [13]; oxidized *A. vinelandii* flavodoxin 10600 M^{-1} cm^{-1} at 450 nm [12]; *M. elsdenii* flavodoxin semiquinone, 4500 M^{-1} cm^{-1} at 580 nm; *A. vinelandii* flavodoxin semiquinone, 5300 M^{-1} cm^{-1} at 580 nm; methyl viologen semiquinone, 13600 M^{-1} cm^{-1} at 604 nm [16]. The midpoint potentials used for the redox couple flavodoxin hydroquinone/semiquinone at pH 7.0 were: *A. vinelandii*, E_m = −495 mV; *M. elsdenii*, E_m = −378 mV [16]. For the redox couple methyl viologen semiquinone/oxidized, −446 mV was used at pH 7.0 [16]. Redox potentials with the redox couple dithionite/sulphite were set according to Mayhew and Van Dijk et al. [16–18].

The contamination of dithionite (from Merck) by sulphite was determined as described by Van Dijk and Veeger [18]. The contamination was found to be 20% and this amount was taken into account for the calculation of the redox potential. The dependence of the pK'_a of (bi)sulphite on the ionic strength was determined experimentally. An unbuffered solution of sodium sulphite was titrated under argon with HCl at different NaCl concentrations. The pK'_a followed the Debye-Hückel law in the following way: up to 100 mM NaCl, the pK'_a at 30 °C remained unchanged (pK'_a = 6.95); above 100 mM NaCl up to 600 mM NaCl it was found that $pK'_a = pK'_a - 0.65 \sqrt{I} + 0.16\, I$.

The samples for the EPR experiments were prepared at 25 °C under argon; the EPR tubes were filled under argon and the samples were manually frozen in isopentane at −100 °C.

EPR spectra were taken on a Bruker ER 200D spectrometer, connected to a Data General NOVA-3 computer in

which all spectra were stored. Baseline corrections were made by fractional subtractions of the signal of a solution of a standard buffer taken separately in the same calibrated tube. Temperature control at low temperature was kept by a home-built He system as described by Lundin and Aasa [19]. The temperature was measured with a carbon resistor (Allen Bradley) of about 5.6 kΩ at room temperature. All spectra were taken at 10.5 ± 0.5 K.

The intensity of the signal was calculated from the amplitude of the g_y peak of all signals. The integrated intensities of corrected spectra were compared with a 1 mM Cu^{2+} solution in 2 mM sodium perchlorate at pH 2.0. The EPR tubes used were calibrated with a standard spin label (4-maleimido-2.2.6.6 tetramethylpiperidine-1-oxyl) solution in dioxane.

It was ascertained that no significant change in redox potential occurred due to freezing. A reduced methyl viologen solution at −440 mV in the standard incubation mixture was manually frozen (in isopentane at −100 °C) and the signal was measured. No significant difference in amplitude was observed.

The microwave frequency was measured in each experiment with a counter (Systrom, Donner counter 1017). The field sweep and central position were calibrated by using an NMR probe. In the text the EPR settings are given. A sweep width of 1000 G (0.1 T) is in fact 993.0 G (0.0993 T) and the central values are respectively 3490.6 and 3387.0 for 3500.0 and 3400.0.

Materials

ATP, creatine kinase (EC 2.7.3.2), creatine phosphate, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) and bovine serum albumin were obtained from Sigma Chemical Co; all other chemicals were of the highest commercial grades. Argon was purified by passage over hot (150 °C) BASF catalyst. Protein concentration was estimated with the Lowry method and according to Sedmak and Grossberg [20].

RESULTS

Fig. 1 shows the results of the effect of the redox potential of the redox couple dithionite/sulfite on the nitrogenase activity. Not $S_2O_4^{2-}$ but its dissociation product, $SO_2^{\cdot-}$ is the actual electron donor for nitrogenase [21,22]. In all experiments the concentration of $S_2O_4^{2-}$ was kept constant at 5 mM. With a dissociation constant for dithionite of 1.4 nM, it can be calculated that the concentration of $SO_2^{\cdot-}$ = 2.6 μM. The redox potential was increased by adding the oxidation product of $SO_2^{\cdot-}$, Na_2SO_3 [16,17]. It is clear that the nitrogenase reaction is independent of the redox potential up to potentials of −440 mV. At higher potentials and at higher ionic strength the nitrogenase activity declines and at potentials above −365 mV and an ionic strength of 950 mM NaCl hardly any nitrogenase activity is detectable. It was ascertained that at low potentials a total concentration of 2.6 μM $SO_2^{\cdot-}$ (5 mM $S_2O_4^{2-}$) is saturating in the nitrogenase reaction. Since during variation of the Na_2SO_3 concentration those of $SO_2^{\cdot-}$ and Av_2 are not changed it can be assumed that the rate of reduction of oxidized Av_2 by $SO_2^{\cdot-}$ is the same at high and at low potentials, although assuming equilibrium between the $SO_2^{\cdot-}$ $SO_3^{\cdot-}$ and Av_2ox/Av_2red couples at higher redox potentials Av_2 becomes more oxidized.

The observed inhibition could be due to an effect of the redox potential or could be due to an increase in ionic strength.

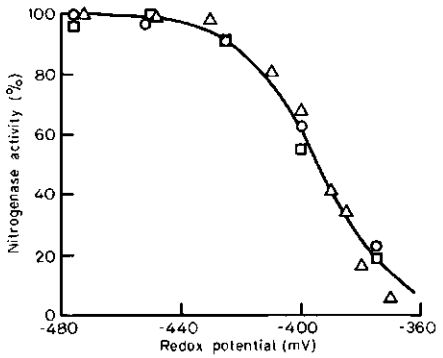


Fig. 1. *The effect of the applied redox potential on the activity of different nitrogenase complexes.* Redox potentials were set with $Na_2S_2O_4$ (5 mM) and varying amounts of Na_2SO_3. The nitrogenase activity was followed by the H_2 production. (○—·—○) $[Av_1]$ = 0.33 μM, $[Av_2]$ = 0.33 μM, 100% activity = 30 μM H_2 produced · min^{-1}; (□ ··· □) $[Av_1]$ = 0.07 μM, $[Av_2]$ = 0.96 μM, 100% activity is 30 μM H_2 produced · min^{-1}; (Δ ··· Δ) $[Av_1]$ = 0.11 μM, $[Av_2]$ = 2.9 μM, 100% activity is 48.4 μM H_2 produced · min^{-1}

For instance, to set a redox potential of −395 mV, 113 mM Na_2SO_3 was added, resulting in a 50% ± 8% inhibition of the nitrogenase activity independently of the type of nitrogenase complex tested (Fig. 1). When 100 mM Na_2SO_4 was added, giving the same extra ionic strength, the activity was inhibited by 37% ± 7%. At an applied redox potential of −377 mV, with 226 mM Na_2SO_3 added, the nitrogenase activity is 70% inhibited. The same inhibition was found when 200 mM Na_2SO_4 was added to give a similar ionic strength. Since we cannot discriminate between the effects of redox potential and ionic strength on the nitrogenase activity, we used a different method to study the effect of redox potential on the nitrogenase activity. In this method substrate amounts of photochemically reduced low-potential electron carriers are oxidized by nitrogenase. In all cases the same dependence of activity on applied redox potential is found. From a single progress curve the nitrogenase activity at a series of redox potentials can be calculated (Fig. 2). The nitrogenase activity was also monitored polarographically in the same cuvette (see Materials and Methods). *Azotobacter vinelandii* and *Megasphaera elsdenii* flavodoxins, methyl viologen and benzyl viologen were tested in the concentration range from 25 μM to 350 μM.

A typical progress curve for the *M. elsdenii* flavodoxin and methyl viologen are given in Fig. 2. After the addition of the nitrogenase components and deazaflavine-sulphonic acid the electron carriers were reduced with a short intense light pulse. Nitrogenase was allowed to oxidize the reduced carriers. To be able to calculate the redox potential of the electron carriers, the total amount of carrier and the amount of reduced or oxidized carrier must be known. To determine the concentration of reduced methyl viologen or flavodoxin semiquinone it is important to know the contribution of both species to the measured absorption. We therefore ran controls without the electron carriers added. These controls are given in Fig. 2 as dotted lines. From a number of assays the average E_m and n

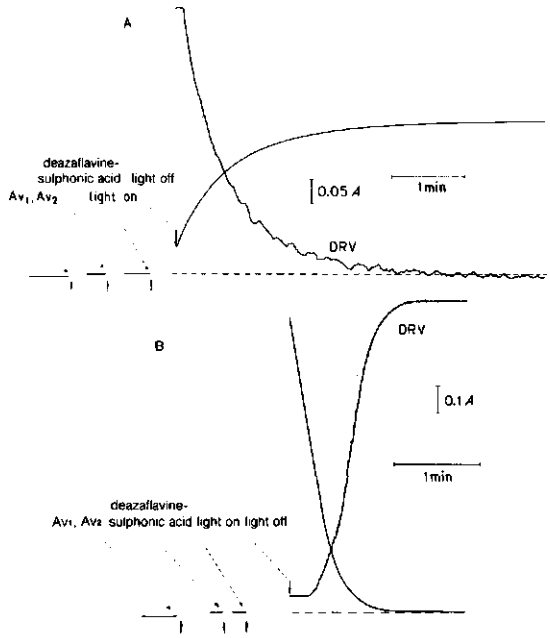


Fig. 2. *Oxidation of photochemically reduced methyl viologen and* M. elsdenii *flavodoxin by nitrogenase.* (A) The reaction mixture contained 100 μM methyl viologen. At the arrows, 0.3 μM Av_1, 0.3 μM Av_2 and 125 μM deazaflavine-sulphonic acid were added. After the light pulse the decrease in absorption of methyl viologen semiquinone at 580 nm was plotted. The calculated first-order derivative was also plotted (DRV); 100% nitrogenase activity was 52 μM H_2 · produced · min^{-1}. (B) The reaction mixture contained 148 μM *M. elsdenii* flavodoxin. At the arrows, 0.24 μM Av_1, 0.24 μM Av_2 and 125 μM deazaflavine-sulphonic acid were added. After the light pulse the production of *M. elsdenii* flavodoxin semiquinone was followed at 580 nm. The calculated first-order derivative was also plotted (DRV); 100% nitrogenase activity was 40 μM H_2 produced · min^{-1}

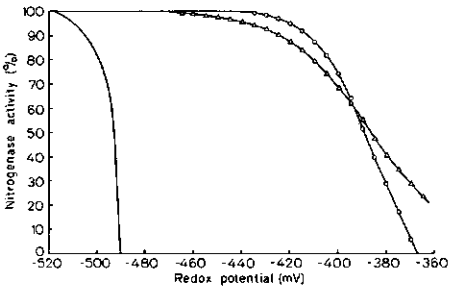


Fig. 3. *The dependence of the nitrogenase activity on the redox potential of* A. vinelandii *flavodoxin,* M. elsdenii *flavodoxin and methyl viologen.* The curves were constructed from time and *n* values from at least six independent measurements as described in Materials and Methods. (· · ·) 73–336 μM *A. vinelandii* flavodoxin, 0.1 μM Av_1 and 0.1 μM Av_2; 50% nitrogenase activity at −500 ± 16.5 mV; 100% nitrogenase activity was 20 μM H_2 produced · min^{-1}. (O– –O) 50–200 μM *M. elsdenii* flavodoxin, 0.25 μM Av_1 and 0.25 μM Av_2; 50% nitrogenase activity at −389 ± 7.7 mV, *n* value 2.49 ± 0.27; 100% nitrogenase activity was 40 μM H_2 produced · min^{-1}. (Δ · · · Δ) 50–100 μM methyl viologen; 0.3 μM Av_1 and 0.3 μM Av_2; 50% nitrogenase activity at −386 ± 10 mV, *n* value 1.52 ± 0.19; 100% nitrogenase activity was 52 μM H_2 produced · min^{-1}

values were calculated. These values were used to construct activity versus redox potential curves as shown in Fig. 3. The results of *A. vinelandii* flavodoxin hydroquinone oxidation show that this flavodoxin hydroquinone is not oxidized beyond −480 mV. At the highest flavodoxin concentration tested (366 μM), with a midpoint potential of −495 mV at pH 7.0, it can be calculated that at −480 mV the flavodoxin hydroquinone concentration is 132 μM. However under continuous illumination flavodoxin concentrations around 10 μM give maximum activity [7,23,24]. We can conclude that *A. vinelandii* flavodoxin semiquinone acts as an inhibitor and that it is therefore not possible to use this flavodoxin as an electron donor and as an indicator to study the redox potential dependence of the nitrogenase reaction.

Benzyl viologen cannot be used as electron donor and indicator because at concentrations above 25 μM it inhibits the nitrogenase activity strongly and at lower concentrations a considerable amount of the semiquinone form binds strongly to the nitrogenase proteins. Methyl viologen is also inhibitory but only at concentrations above 200 μM. At 50 μM and 100 μM methyl viologen the tested nitrogenase activity and the shape of the activity/redox potential curve was found, within experimental error, to be independent of the methyl viologen concentration.

With *M. elsdenii* flavodoxin we found, within the concentration range tested (50–200 μM), no significant effect of the flavodoxin concentration on either the nitrogenase activity or on the activity/redox potential curve.

In order to verify that the drop in nitrogenase activity at higher potentials is not due to non-equilibrium between Av_2 and the electron donor, we varied the absolute and relative concentrations of Av_2 and electron donor. The activities of a 1:1 and a 1:24 complex show, within the experimental error, the same dependence on the applied redox potential. It is important to note that these reconstituted complexes have different specific activities. In a 1:1 complex the amount of Av_2 is thought to limit the overall activity, but in the 1:24 complex the flux of electrons from reductant via Av_2 to Av_1 is thought to be maximal.

During the oxidation of the reduced electron donor by nitrogenase the concentration of the reduced electron donor diminishes. It is therefore important to be sure that the concentration of electron donor does not become rate-limiting at higher potentials. From its midpoint potential one can calculate the concentration of electron donor at all potentials of the progress curve. At −370 mV, at a total concentration of 100 μM methyl viologen and 200 μM *M. elsdenii* flavodoxin, the concentrations of the reducing species are 5.1 μM and 87 μM respectively. Since we tested different concentrations of methyl viologen and *M. elsdenii* flavodoxin (50–200 μM) in relation to redox potential and we found no significant changes in the shape or the position of the nitrogenase activity curves, we concluded that under our conditions, even with the 1:1 complexes, the amount of electron donor was not rate-limiting. We also tested the dependence on the redox potential with photochemically reduced flavodoxins of *A. vinelandii*

Table 1. g *values of nitrogenase Fe proteins*
EPR spectra were recorded and g values derived as described in Materials and Methods. For details see Fig. 3

Fe protein	g_x	g_y	g_z	n	Midpoint potential	References
					mV	
Av_2(Mg)	1.864	1.941	2.048	2	− 393 (pH 7.0)	this work
Av_2(MgADP)	1.874	1.947	2.051	1	− 473 (pH 7.0)	this work
Av_2(MgATP)	1.895 − 1.90	1.940	2.045	2	− 435 (pH 7.0)	this work
Ac_2(Mg)	1.87	1.94	2.05	–	–	[10]
Kp_2(Mg)	1.865	1.942	2.053	–	–	[26]
Kp_2(MgATP)	–	1.929	2.036	–	–	[26]
Cp_2(Mg)	1.87	1.94	2.06	1	− 294 (pH 7.5)	[8, 25]
Cp_2(MgATP)	–	1.93	2.04	1	− 400 (pH 7.5)	[8, 25]
Cp_2(MgADP)	–	1.93	2.04	1	− 380 (pH 7.5)	[8, 25]

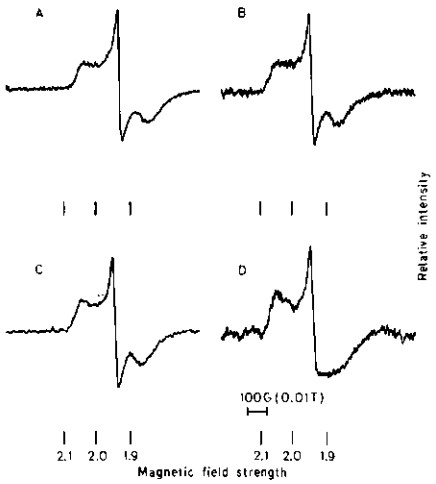


Fig. 4. *Electron paramagnetic resonance spectra of reduced* Av_2. Samples were prepared as described in Materials and Methods. (A) 56 μM Av_2, 5 mM $Na_2S_2O_4$, 1 mM Na_2SO_3, 7.5 mM $MgCl_2$, 112 mM Hepes/KOH, final pH 7.0, final redox potential − 520 mV; specific instrument settings: gain 3.2×10^5; microwave power 1.28 mW; temperature 10.7 K, central field 3400, microwave frequency 9.41865 GHz. (B) 21.5 μM Av_2, 5 mM $Na_2S_2O_4$, 1 mM Na_2SO_3, 7.5 mM $MgCl_2$, 5 mM ADP, 112 mM Hepes/KOH final pH 7.8, final redox potential − 600 mV; specific instrument settings: gain 5×10^5; microwave power 0.51 mW; temperature 9.6 K, central field 3500; microwave frequency 9.42216 GHz. (C) 56 μM Av_2, 5 mM $Na_2S_2O_4$, 1 mM Na_2SO_3, 7.5 mM $MgCl_2$, 5 mM ADP, 112 mM Hepes/KOH, final pH 7.0, final redox potential − 520 mV; specific instrument settings: gain 2.5×10^5; microwave power 1.28 mW; temperature 10.7 K; central field 3400; microwave frequency 9.42163 GHz. (D) 31.5 μM Av_2, 5 mM $Na_2S_2O_4$; 1 mM Na_2SO_3, 7.5 mM $MgCl_2$, 5 mM ATP, 112 mM Hepes/KOH, final pH 7.0, final redox potential − 520 mV; specific instrument settings: gain 5×10^5; microwave power 1.28 mW; temperature 10.7 K; central field 3500; microwave frequency 9.4089 GHz. In all experiments sweep width 100 G (0.01 T)/min; scan time 200 s, time constant 0.5 s and the modulation amplitude 10 G (0.001 T)

and *M. elsdenii* as well as with methyl viologen semiquinone at pH 8.0. Although the maximum nitrogenase activity was between 50 − 70% of that at pH 7.0, the form of the activity/redox potential curves were similar to those as at pH 7.0.

The steady-state experiments indicate that we have made our measurements under conditions of redox equilibrium between free Av_2 and the electron donor. Under these conditions it is demonstrated that limitations in the value of the redox potential lead to a rate-limiting step in the nitrogenase reaction showing a decline in activity at redox potentials above − 440 mV.

From the results of Fig. 3, it cannot be derived which of the two nitrogenase components is responsible for this redox behaviour. Although in different concentrations and ratios, both components need to be present for activity and redox behaviour. Since the redox properties of Av_1 are much more difficult to study than those of Av_2, the latter was chosen for studying its redox behaviour.

Redox titrations have been performed on Cp_2 in the absence and presence of MgADP and MgATP [8]. Although Zumpft et al. found deviation from an $n = 1$ redox-titration behaviour, they nevertheless concluded that Cp_2 takes up one electron. In order to elucidate which component is involved, we performed redox titrations of Av_2 with the redox couple $SO_2^{\cdot-}$ SO_3^{2-} and measured the oxidation-reduction state of the 4Fe-4S cluster by EPR spectroscopy. Because we found that the EPR spectra of Av_2 isolated from our strain differ considerably from the data published, the EPR spectra of Av_2, Av_2 + MgATP and Av_2 + MgADP are given in Fig. 4. The g values of the Fe protein are given in Table 1.

It was checked that at high Na_2SO_3 concentrations, the shape of the EPR curve was not changed. The EPR spectrum of Av_2 + MgADP is clearly different from that of Av_2 + MgATP, in contrast to Cp_2 [8, 25]. At low MgADP concentrations the Cp_2 spectrum does not change, although it shifts slightly. But at higher MgADP concentrations, the EPR spectrum resembles that of Cp_2 + MgATP [8, 25]. With Av_2 in the presence of 5 mM MgADP the second shift leading to an ATP-type of spectrum, as observed with Cp_2, does not occur. Furthermore the EPR spectrum in the presence of MgATP is much less axial than in Cp_2 and Kp_2 [8, 25, 26]. Apparently the shift in g_x is dependent on the species and might reflect the extent of conformational changes occurring upon binding of MgATP.

We used the height of the g_y peak as a measure of the amount of Av_2 in the reduced state. Fig. 5 shows the influence of the applied redox potential on the height of this signal. Three important redox features of Av_2 are notable. The experimental points of the redox titration of Av_2 and Av_2 + MgATP fit a theoretical curve for a two-electron redox reaction better than an $n = 1$ curve with an $E_m = -435$ mV. The midpoint potential of Av_2 without adenine nucleotides

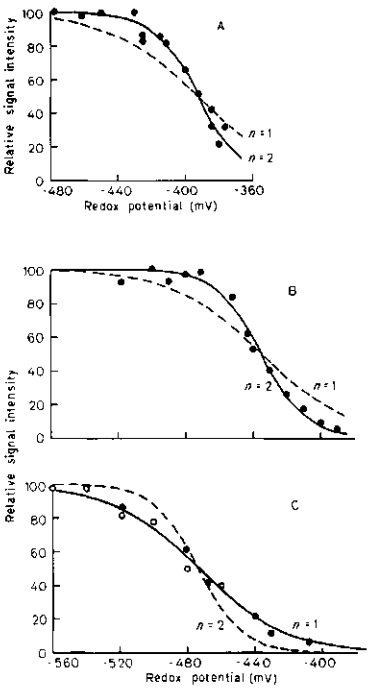


Fig. 5. *Dependence of the electron paramagnetic resonance signal of Av_2 in the presence or absence of Mg^{2+}-adenine nucleotides on the redox potential.* The redox potential was set with $Na_2S_2O_4$ and Na_2SO_3 and samples were prepared and frozen as described in Materials and Methods. Three different protein preparations gave the same results. (A) (● - -●) 56 μM Av_2, 5 mM $Na_2S_2O_4$, 7.5 mM $MgCl_2$, 112 mM Hepes/KOH and varying amounts of Na_2SO_3 to obtain the redox potential indicated, final pH 7.0. Instruments settings as in Fig. 4A. The $g = 1.941$ signal is plotted against the redox potential. The dotted line is the theoretical curve for a one-electron transition, the solid line for a two-electron transition with an $E_m = -393$ mV. According to the chi-squared test the data lie on a theoretical $n = 2$ curve, with an $E_m = -393$ mV and a reliability of 60%. In contrast the reliability of the hypothesis that the data fit an $n = 1$ curve is 2.5%. (B) (● - -●) 31.5 μM Av_2, 5 mM $Na_2S_2O_4$, 7.5 mM $MgCl_2$, 5 mM ATP, 112 mM Hepes KOH and varying amounts of Na_2SO_3 to obtain the redox potential indicated, final pH 7.0. Instrument settings as in Fig. 4D. The $g = 1.940$ signal is plotted against the redox potential. The dotted line is the theoretical curve for a one-electron transition, the solid line for a two-electron transition with $E_m = -435$ mV. According to the chi-squared test the data lie on a theoretical $n = 2$ curve with an $E_m = -435$ mV and a reliability of 97.5%. (C) (● - -●) 56 μM Av_2, 5 mM $Na_2S_2O_4$, 7.5 mM $MgCl_2$, 5 mM ADP, 112 mM Hepes/KOH, and varying amounts of Na_2SO_3 to obtain the redox potential indicated, final pH 7.0. The instrument settings as in Fig. 4C. The $g = 1.947$ signal is plotted against the redox potential. The normalized maximum signal intensity at −600 mV at pH 7.8 was taken as 100%. (○ - -○) 21.5 μM Av_2, 5 mM $Na_2S_2O_4$, 7.5 mM $MgCl_2$, 5 mM ADP, 112 mM Hepes/KOH and varying amounts of Na_2SO_3 to obtain the redox potential indicated, final pH 7.8. Instrument settings as in Fig. 4B. The $g = 1.947$ signal is plotted against the redox potential. The solid line is the theoretical curve for a one-electron transition, the dotted line for a two-electron transition with an $E_m = -473$ mV. According to the chi-squared test the data lie on a theoretical $n = 1$ curve with an $E_m = -473$ mV and a reliability of 95%.

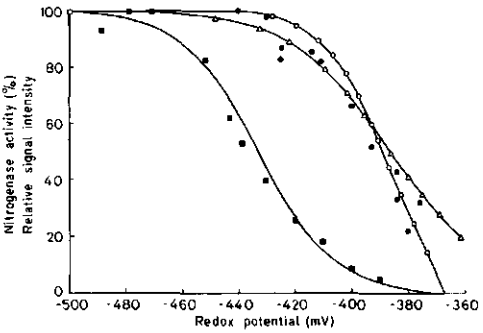


Fig. 6. *Dependence of the redox state of Av_2 and of the nitrogenase activity on the redox potential.* The state of reduction of Av_2 was measured with EPR spectroscopy. The nitrogenase activity was measured by dye oxidation. (Δ - -Δ) Activity of a 1:1 nitrogenase complex with methyl viologen as electron donor; (○ - -○) activity of 1:1 and 1:24 nitrogenase complexes with *M. elsdenii* flavodoxin as electron donor (Fig. 3); (● - -●) relative $g = 1.941$ signal intensity of $Av_2 + MgCl_2$ at the redox potentials indicated (Fig. 4A); (■ - -■) relative $g = 1.940$ signal intensity of Av_2 + MgATP at the redox potentials indicated (Fig. 4B)

is considerably more negative than that of Cp_2 and the shift in midpoint potential by MgATP is considerably less (Table 1). A further difference is that with Av_2 the midpoint potential in the presence of MgADP is more negative than that of Cp_2 + MgADP [8] (Table 1).

The intensities of the three signals when compared with a standard Cu^{2+} solution integrate at the lowest potentials (−520 mV at pH 7.0 for Av_2 and Av_2 + MgATP and at −600 mV for Av_2 + MgADP at pH 7.8) to around one electron per active Av_2 molecule. It has already been noted that these EPR signals are highly temperature-dependent. We measured the intensity of the signals at both 9.6 K or at 10.7 K. At neither temperature was a difference in intensity found, but at 13.2 K the signal significantly declines.

Fig. 6 shows the oxidation reduction state of $Av_2 + Mg^{2+}$ in the presence and absence of ATP, as measured by EPR spectroscopy and the activity of the nitrogenase at different redox potentials. It is clear that the activity of nitrogenase follows the oxidation reduction state of Av_2 without adenine nucleotides bound more closely than the oxidation reduction state of Av_2 plus MgATP.

DISCUSSION

Our results indicate quite convincingly that Av_2, considered to be a [4Fe-4S]-cluster-containing protein, shows a redox behaviour characteristic for a two-electron transferring protein. This behaviour is found in the 'oxidized' protein, as *isolated, as well as in its MgATP complex*; *but not in its* MgADP complex, which shows a one-electron transfer redox behaviour. Our data are in contrast to studies with the Fe protein from *Clostridium pasteurianum*, where a one-electron redox transfer was found, irrespective of the binding of Mg^{2+} adenine nucleotides [8, 25]. Furthermore Ljones and Burris [27] concluded from physiological oxidation experiments of Cp_2 by Cp_1 that a one-electron transfer takes place.

On the other hand Watt and Bulen [6] could not decide whether the nitrogenase activity/redox potential curve of the three-component complex of *Azotobacter vinelandii* followed an $n = 1$ or $n = 2$ pattern. With methyl viologen as electron donor we found n values between $n = 1$ and $n = 2$. With *Megasphaera elsdenii* flavodoxin a value above 2 is significant. Evans and Albrecht [5] concluded that crude nitrogenase from *Chromatium vinosum* follows an $n = 2$ pattern in its activity curve. From the results it seems to us that the n value found is more or less dependent upon the electron donor used. Interactions of electron donors with Av_2 or with the $Av_2 \cdot Av_1$ complex might be responsible for the different n values.

However, all data indicate that the n value of the nitrogenase activity/vs redox potential is more than one. Thorneley et al. [21] observed in rapid kinetic studies with dye-oxidized Ac_2 that two electrons could be accomodated. The uptake of the first, rapidly reacting electron is associated with the EPR signal; the second slower electron uptake was, as the first electron uptake, spectrally visible. Thorneley et al. [21] concluded that this second electron uptake process is associated with the presence of inactive protein. Later Lowe [28] revised this view in suggesting that in Kp_2 another rapidly relaxing paramagnetic centre is present, in view of the broad anisotropic EPR spectra observed.

Our data on the activity/redox potential dependence, coinciding with the EPR titration of free Av_2 (Fig. 6), are in contrast to some of the current views expressed in kinetic models [1,2,29–31]. Thorneley et al. [1,29] suggested that the rate-limiting step is the dissociation of oxidized Fe protein from the Mo-Fe protein. In addition it was proposed [2,30] that the reduction of oxidized Fe protein must be rate-limiting, since a variable percentage of Fe protein was found to be in its oxidized form [26,32,33]. In the latter experiments, the redox state of the Fe protein was estimated from the relative EPR signal intensity during turn-over conditions [26,32,33]. It is, however, doubtful that one can compare nitrogenase activities with respect to the rate-limiting step at very different protein concentrations. For instance at relatively high protein concentrations, as used for EPR measurements under turnover conditions, the nitrogenase activity is inhibited as compared to more dilute solutions [26] (and D. J. Lowe and R. N. F. Thorneley, personal communication), an observation to be expected from our sequential mechanism described in the Appendix. At high protein concentrations complex formation is favoured (mass balance) and the nitrogenase activity is inhibited [26] even at a relative low concentration of oxidized free Fe protein. In addition it was demonstrated that the bound Fe protein is hardly reducible [30,34]. Using a dissociation constant of 0.1 μM and 1:1 stoichiometry of the complex [35], one can calculate the free Fe protein concentration in turnover EPR experiments (12.5 μM initial concentrations of both proteins gives 10% free Fe protein) and in our kinetic experiments (0.33 μM initial concentrations of both proteins gives 40% free Fe protein). However the amount of reduced Fe protein under turnover conditions at high protein concentration is more than 50% [26,32,33].

Since we found that variation of the concentrations of the various donors does not lead to any significant change in activity at all redox potentials tested, we conclude that the free reducible Fe protein is in redox equilibrium with the electron donors. Neither did we observe any influence on the activity/redox potential profile at the different concentrations of the Fe protein tested.

It has been proposed [31] that the steps prior to the nitrogenase reaction proceed in a random mechanism. In other words, free reduced Fe protein reacts with either the Mo-Fe protein, followed by MgATP binding on the complex or by binding ATP followed by reaction of the MgATP · Fe-protein complex with the Mo-Fe protein. All binding and complex formation proceeds in rapid equilibrium between reactants. Thereafter irreversible electron transfer takes place from the Fe protein to the Mo-Fe protein with subsequent hydrolysis of ATP to ADP and phosphate. The rate-limiting step in the nitrogenase reaction occurs after the electron transfer. It has been suggested that the slow step is the dissociation of the two proteins from each other [1,29].

The activity/redox profiles coinciding with the EPR/redox behaviour of the free Fe protein are in contrast to a random mechanism and require a sequential mechanism. In fact consideration of the data in the literature [1], assuming that nitrogenase components from different species follow the same pattern, makes a random mechanism unlikely. For instance not only is the rate of formation of the complex $Av_2(red) \cdot Av_1(ox)$ very fast, but also the association constant of this complex is very high ($\approx 10^7\ M^{-1}$) and much higher than the association constants of the Fe-protein · MgATP complex ($\approx 5 \times 10^4\ M^{-1}$) [31]. The association constant for the $Av_2(red) \cdot Av_1(ox)$ complex could be in our opinion, an order of magnitude higher, since this value was derived from dilution experiments under steady-state activity, during which MgATP is present [35]. A further consideration for a sequential mechanism is the fact that if the Fe-protein · MgATP complex were reacting with the Mo-Fe protein, an activity/redox profile coinciding with the redox behaviour of the Fe-protein · MgATP complex could be expected, especially for an $Av_2:Av_1$ ratio 1:1, since under the conditions of our experiment 90% of the free Fe protein (40% of the total amount of Av_2) is in this form.

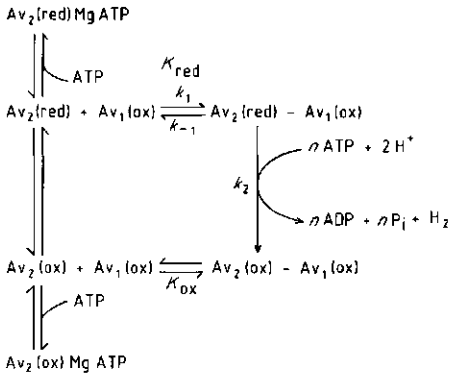
Our results indicate that, under redox potential limitation, catalysis might proceed by two-electron transfer. It is not necessary that the two electrons are donated simultaneously. One electron can be donated rapidly, the second electron then must be donated more slowly. In this case the redox behaviour of the $Av_2 \cdot MgADP$ complex might explain the strong inhibition of the nitrogenase reaction by MgADP. The absence of the second electron prevents catalysis rather than competition with bound MgATP as proposed [36].

APPENDIX

Scheme 1 gives the basic steps, as a minimal hypothesis, necessary to explain the phenomena observed. In the theoretical considerations for an ordered mechanism in terms of the relations with the activity/redox profiles, the following facts and assumptions were taken into account.

a) The concentration of the $Av_2(red) \cdot Av_1(ox)$ complex determines the rate of the reaction, because of the 1000-fold higher affinity of the Fe protein for the Mo-Fe protein than for MgATP.

b) The transfer of electrons in catalysis is irreversible and the rate-limiting step of the reaction occurs somewhere in the whole sequence of events. According to Thorneley and Mortenson [1], this is the rate of dissociation of $Av_2(ox)$ from $Av_2(ox) \cdot Av_1(ox)$, although no clear data have been published.



Scheme 1

c) All equilibrium steps are fast compared with the rate of catalysis.

Since the experiments were carried out at a fixed concentration of Fe protein, the total protein concentration $[Av_2]_0$ is equal to the sum of the concentrations of reduced $[Av_2(red)]$ and oxidized $[Av_2(ox)]$ and their MgATP complexes

$$[Av_2]_0 = [Av_2(red)] + [Av_2(red)MgATP] + [Av_2(ox)] + [Av_2(ox)MgATP] . \quad (1)$$

The relation

$$K^T = \frac{[Av_2(red)]\,[MgATP]}{[Av_2(red)MgATP]}$$

leads to

$$[Av(red)MgATP] = [Av_2(red)]c_1 \quad (2)$$

in which K^T is the dissociation constant of $Av_2(red)MgATP$ and $c_1 = [MgATP]/K^T$. Similarly

$$[Av_2(ox)MgATP] = [Av_2(ox)]c_2 \quad (3)$$

where $c_2 = [MgATP]/K^o$ and K^o is the dissociation constant of $Av_2(ox)MgATP$.

Under conditions that the applied redox potential is much lower than the midpoint potential of the $Av_2(red)/Av_2(ox)$ couple

$$[Av_2]_0 = [Av_2(red)] + [Av_2(red)MgATP]$$

which in combination with Eqn (2) leads to

$$[Av_2(red)] = \frac{[Av_2]_0}{1 + c_1} . \quad (4)$$

Furthermore under these conditions, taking into account Eqn (4)

$$r = \frac{V}{1 + \dfrac{K_{red}}{[Av_2(red)]}} = \frac{V}{1 + \dfrac{K_{red}(1 + c_1)}{[Av_2]_0}} \quad (5)$$

where K_{red} is the dissociation constant of $Av_2(red) - Av_1(ox)$ and $k_{-1} \gg k_2$.

At redox potentials where $Av_2(ox)$ is present, Eqn (5) modifies to:

$$r_i = \frac{V}{1 + \dfrac{K_{red}}{[Av_2(red)]}\left(1 + \dfrac{[Av_2(ox)]}{K_{ox}}\right)} . \quad (6)$$

Taking into account the relation

$$[Av_2(red)] = [Av_2]_0 - [Av_2(red)MgATP] - [Av_2(ox)] - [Av_2(ox)MgATP]$$

which in view of Eqns (2) and (3) leads to

$$[Av_2(red)] = \frac{[Av_2]_0 - [Av_2(ox)](1 + c_2)}{(1 + c_1)} \quad (7)$$

Eqn (6) modifies to

$$r_i = \frac{V}{1 + \dfrac{K_{red}(1 + c_1)}{[Av_2]_0 - Av_2(ox)(1 + c_2)}\left(1 + \dfrac{[Av_2(ox)]}{K_{ox}}\right)} \quad (8)$$

in which K_{ox} is the dissociation constant of $Av_2(ox) \cdot Av_1(ox)$ and r_i is the remaining activity.

Dividing Eqn (8) by Eqn (6) gives the following relation:

$$\frac{r_i}{r} = \frac{1 + \dfrac{K_{red}(1 + c_1)}{[Av_2]_0}}{1 + \dfrac{K_{red}(1 + c_1)}{[Av_2]_0 - [Av_2(ox)](1 + c_2)}\left(1 + \dfrac{[Av_2(ox)]}{K_{ox}}\right)} \quad (9)$$

The redox relation

$$\Delta E = -\frac{RT}{nF} \ln \frac{[Av_2(red)]}{[Av_2(ox)]} \quad (10)$$

leads, by substitution of Eqns (1–3), to

$$[Av_2(ox)] = \frac{[Av_2]_0}{(1 + c_2) + (1 + c_1)\exp(-p)} \quad (11)$$

with $p = nF\Delta E/RT$ and $\Delta E = E - E_0$, in which E is the applied redox potential and E_0 the midpoint potential of the free Av_2 redox couple.

Substituting Eqn (11) into Eqn (9) and rearranging leads to:

$$\frac{r_i}{r} = \frac{1 + \dfrac{K_{red}(1 + c_1)}{[Av_2]_0}}{1 + \dfrac{K_{red}(1 + c_1)}{[Av_2]_0}\left\{1 - \dfrac{(1 + c_2)}{(1 + c_2) + (1 + c_1)\exp(-p)}\right\}}\left(1 + \frac{[Av_2]_0}{K_{ox}\{(1 + c_2) + (1 + c_1)\exp(-p)\}}\right) \quad (12)$$

or after rearrangement

$$\frac{v_i}{v} = \frac{1}{1 + \frac{K_{red}}{K_{ox}} \left\{ \frac{K_{ox}(1 + c_2) + [Av_2]}{K_{red}(1 + c_1) + [Av_2]} \right\} \exp(+p)} \quad (13)$$

or

$$\frac{v_i}{v} = \frac{1}{1 + a \exp(nF\Delta E/RT)} \quad (14)$$

where

$$\Delta E = E - E_0',$$

$$E_0' = E_0 - \frac{RT}{nF} \ln a \text{ and } E_0' = \text{apparent midpoint potential.}$$

Eqn (14) fits the observed activity/redox potential profile only when $a = 1$. In that case it is similar to Eqn (10), the Nernst equation. The potential E_0', at which the nitrogenase activity is 50%, is related to the E_0 of the redox reaction involved in the rate-limiting step. This factor contains equilibrium constants and $[Av_2]_0$. Experimentally it was found that, within the concentration range of 0.1–2.0 μM of Av_2 tested, no effect upon the E_0' was detectable. It can be concluded that, in view of the observation that $E_0' \approx E_0$, $a = 1$ can only be obtained when $K_{red} \approx K_{ox}$ and $c_1 \approx c_2$ thus $K' \approx K^\circ$.

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CHAPTER III. Is Fe-protein of nitrogenase a one or a two electron donor/acceptor?

Introduction

Before 1976, it was not clear whether the Fe-protein of nitrogenase had to be considered as a one- or as a two-electron donor/acceptor. Mortenson *et al.* [1] reported that the Fe-protein of *Clostridium pasteurianum* (Cp_2), with a specific activity of 2300 nmoles C_2H_2 reduced.min^{-1}.mg^{-1} and an iron content of 3.6 Fe atoms/molecule, donates 1.7 electrons/molecule upon oxidation by dyes. Less active preparations donated 0.9 electron/molecule. It was also stated that there was no difference between the dye oxidized Fe-protein and the physiologically oxidized Fe-protein with respect to their spectra and redox properties. They also showed redox titrations from which it was concluded that Cp_2 behaved as a one electron redox protein, although the data deviated from the theoretical n=1 curve. In fact, one can calculate from their redox titrations in the presence of MgATP and MgADP an n-value of 1.7.

After 1976 there was no further discussion about the redox behaviour of Fe-protein. It was generally accepted that Fe-protein acts as a one electron donor/acceptor. The avaiblable data indicating the presence of a one electron accepting/donating redox center in Fe-protein were summarized by Orme-Johnson *et al.* [2].

I. Generally 3-4 iron and sulphide atoms were found per molecule Fe-protein [3-6].

II. Cluster extrusion experiments [7,8], Mössbauer [9,10], EPR [11-14] and MCD [15] experiments all suggest the presence of a [4Fe-4S] cluster as found in ferredoxins.

III. Linear Electric Field Effects (LEFE) experiments were performed on Fe-protein. In these experiments the extent of the shift of the g-values of an EPR active center is measured when a strong electric field is applied to the sample (10^4-10^5 V/cm). The results of these experiments with reduced Fe-protein in the presence and absence of MgATP, are consistent with the presence of a [4Fe-4S] cluster [16].

IV. Redox titrations performed with Fe-protein were consistent with an n=1 redox behaviour. The degree of reduction was monitored by EPR spectroscopy and by the absorbance at 425 nm [17].

V. Thorneley et al. [18] reported stopped-flow studies with Fe-protein. The reduction of phenazine methosulfate oxidized Ac_2 by dithionite was monitored by the absorbance at 425 nm and four phases were distinguished. The initial rapid step accounts for the uptake of about one electron per molecule Ac_2 and for a second electron in the subsequent three steps. The uptake of the second electron was considered to be too slow to be of any importance during turnover. Oxygen-damaged Fe-protein bleaches in slow steps upon reduction comparable to the last three steps in the reduction of active Fe-protein. So these last three steps were ascribed to inactive protein.

In 1978, Ljones and Burris [19] confirmed the general view that Fe-protein is a one electron donor/acceptor. They demonstrated that physiologically oxidized Fe-protein could be fully reduced by one electron. So when our group started in 1980 to work on the enzymology of nitrogenase, we also regarded Fe-protein as a one-electron donor/acceptor. Only one feature of Fe-protein was unexplained at that time; the integration of the EPR signal yields only about 0.3 electron per molecule [11-14], which is far beyond the expected n=1 value.

Materials and Methods

Enzyme preparations and assay measurements.

Isolation of the nitrogenase proteins and standard activity assays were run as described earlier [20]. The assay mixture contained: 50 mM Hepes/KOH, 10 mM ATP, 15 mM $MgCl_2$, 10 mM creatine phosphate, 0.3 mg creatine kinase/ml, 1 mg bovine serum albumin/ml, final pH 7.5.

Assay mixtures and solutions were made anaerobic by evacuating and filling with argon. To remove the last traces of oxygen the mixtures were flushed for at least 5 min. Dithionite was added from a stock solution (200 mM in 25 mM Tris/HCl, final pH = 7.5), prepared freshly daily, to a final

concentration of 20 mM. Acetylene was added to a final concentration of 20% (v/v). The specific activity of the Av_2 preparations used, was 1700 nmoles C_2H_2 reduced.min^{-1}.mg Av_2^{-1}. The specific activity of Av_1 preparations used was 2000 nmoles C_2H_2 reduced.min^{-1}.mg Av_1^{-1}.

Oxidation of Av_2 was performed on a Bio-Gel P-6DG column (1x8 cm) by running Av_2 through a layer of phenazine methosulfate (0.5 ml of 20 mM) and subsequent desalting as described by Yates et al. [21]. Physiological oxidation was achieved by small amounts of Av_1 in the presence of an ATP-regenerating system. The molecular mass of Av_2 was taken as 63 kDa for the dimer, as determined by amino acid analysis [22,23].

Analytical methods:

Electron counting was performed by means of a stopped flow apparatus. The stopped flow apparatus used was an Update System 1000, Update Instruments Inc., Madison, Wisconsin, U.S.A. The displacement was 5 mm and the ram velocity was 4 cm.$sec.^{-1}$. Each shot produced 0.4 ml. The dead volume of the mixing chamber and the (bi)sulphite stock solutions were calibrated with a reaction mixture containing 100 mM MES/KOH pH = 6.2; 1 mg/ml glucose oxidase; 40 mM glucose, 150 µM methylviologen and 10 mM dithionite. The (bi)sulphite was added and the redox potential calculated from the methyl viologen absorption at 604 nm. In this way also the (bi)sulphite contamination of the dithionite was determined. Physiological oxidation studies and redox reactions were performed in cuvets flushed with argon and closed with rubber stoppers. Absorption measurements were performed on a Zeiss spectrophotometer type M4QIII, PI-2. Protein concentration was determined with the biuret method [26], with bovine serum albumine as standard. The bovine serum albumine concentration was determined by its absorption at 279 nm, ϵ= 44670 $M^{-1}.cm^{-1}$.

Chemicals and gasses

Argon was purified by passage over hot (150°C) BASF catalyst and by sparging through an oxygen scrubbing solution. This solution was reduced by illumination. The solution contained: 100 mM Tris/HCl, 100 mM EDTA, 1 mM

methyl viologen and proflavin till saturation; final pH = 10. Phenazine methosulfate and methyl viologen were obtained from Sigma. All other chemicals were from the highest purity commercially obtainable.

Results and Discussion

The intensity of the EPR signal as a function of redox potential indicates that Av_2 undergoes redox processes characterized as an n=2 redox protein, both in the presence and absence of MgATP [20].
This cannot be explained by assuming that the Fe-protein contains one [4Fe-4S] cluster of a type similar as found in ferredoxins. In an attempt to resolve this discrepancy, we studied the number of electrons taken up by PMS-oxidized Av_2 in a stopped flow apparatus (figure 1).

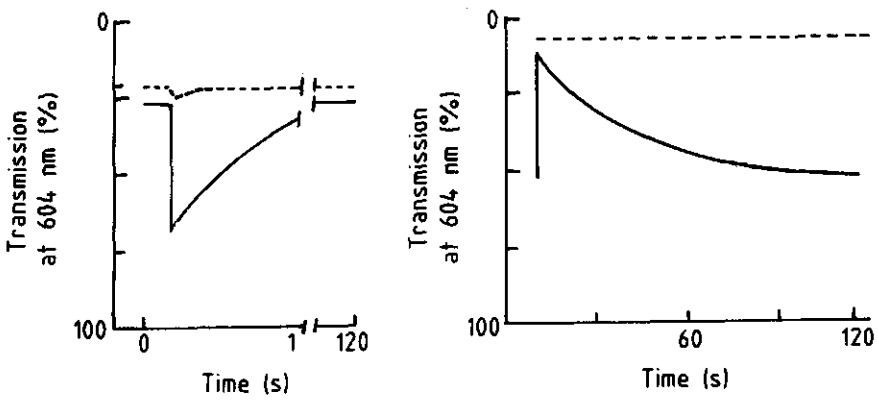

Figure 1. Oxidation of reduced methyl viologen by phenzine methosulphate-oxidized Fe-protein from Azotobacter vinelandii. Transmission changes were recorded at 604 nm in a stopped flow apparatus. The mixing ratio of the two syringes was 1:1. The buffer used was argon flushed 100 mM TES-NaOH, 10 mM $MgCl_2$, final pH 7.0.
A___ Syringe 1 contained buffer plus 100 µM methyl viologen to which, 100 µM $Na_2S_2O_4$ and 20 µM Na_2SO_3 was added. Syringe 2 contained buffer plus 60 µM $K_3Fe(CN)_6$. --- same as above but $K_3Fe(CN)_6$ was omitted from syringe 2.
B___ Syringe 1 contained buffer plus 327 µM methyl viologen to which 150 µM $Na_2S_2O_4$ and 30 µM Na_2SO_3 was added. Syringe 2 contained buffer plus 131 µM Av_2 (specific activity 1700 nmoles C_2H_2 reduced.min^{-1}.$MgAv_2^{-1}$); --- Same as above but Av_2 was omitted from syringe 2.

To establish the validity of the application of this method, a control experiment is shown in figure 1A. In these experiments methyl viologen was used as electron donor and as a redox indicator. One syringe contained 100 mM TES/KOH, pH=7.0, 100 μM methyl viologen, $Na_2S_2O_4$ and Na_2SO_3 were added respectively as 100 μM and 20 μM. The (bi)sulphite is a contamination of the dithionite. In this syringe methyl viologen is reduced by dithionite. The other syringe contained anaerobic 100 mM TES/KOH, pH=7.0 and 60 μM $K_3Fe(CN)_6$. After mixing, a rapid oxidation of methyl viologen occurred. After this fast phase a slow reduction of methyl viologen by the dithionite was seen. The rate limiting step in this reduction is the dissociation of $S_2O_4^{2-}$ into SO_2^- [2]. The dotted line represents a similar experiment but $K_3Fe(CN)_6$ was omitted. From the extent of rapid oxidation of methyl viologen it was calculated that 1.0 ± 0.05 electron was taken up by ferricyanide. Similar experiments with 40 μM and 80 μM ferricyanide also yielded a value of 1.0 ± 0.05 electron per molecule.

It is also possible to calculate the amount of electrons taken up from the difference in final transmission between the experiment with and without electron acceptor. From these final values the redox potential of methyl viologen can be calculated and by assuming redox equilibrium between methyl viologen, dithionite and sulphite, the concentration of dithionite and sulphite can be calculated. The difference of the reduced methyl viologen and dithionite concentrations between the experiments with and without electron acceptor gives the amount of electrons taken up by the electron acceptor. Calculated in this way a value of 0.88 electrons per molecule ferricyanide was obtained.

In figure 1b one syringe contained 100 mM TES/KOH, pH=7.0 and 327 μM methyl viologen which was reduced by the addition of 150 μM dithionite which originally contained 30 μM (bi)sulphite. The other syringe contained 100 mM TES/KOH, pH 7.0 and 131 μM PMS-oxidized Av_2. As is apparent, the oxidation of methyl viologen by Av_2 is rather slow. Redox equilibrium was reached only after two minutes. The calculated final redox potential was -396 mV and the number of electrons taken up was 0.84 electrons per molecule Av_2. When the redox state of the protein was determined by EPR spectroscopy, it was found that at this potential Av_2 is only half-reduced [20]. This means that in case of full reduction, Av_2 takes up 1.7 electrons

per molecule, in agreement with the value found by Mortenson et al. [1] and Thorneley et al. [18]. In contrast with the latter, we observe only a slow phase. Apparently methyl viologen is not a very effective electron donor under these conditions. Surprisingly, Av_2 in the presence of MgATP behaved in a similar manner as Av_2 without MgATP (not shown). Since EPR measurements indicate that in the presence of MgATP the midpoint potential of Av_2 is 40 mV lower (Chapter II), this means that Av_2 can accommodate at least 4 electrons. This prompted us to perform redox titrations upon Av_2-free, Av_2-MgATP and Av_2-MgADP. The redox state of the protein was monitored by the change in absorption at 425 nm.

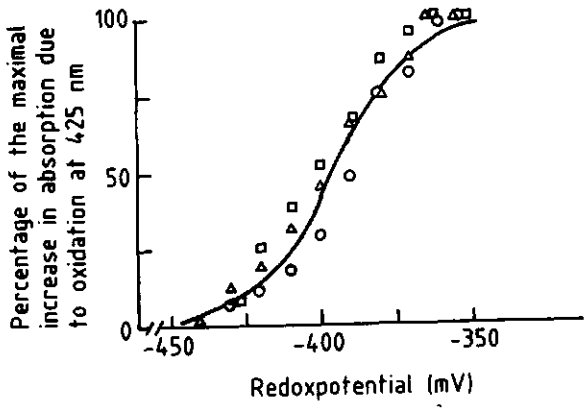

Figure 2. Relationship between the absorbance of Av_2 and the applied redox potential. The redox potential was poised by varying the ratio of the $Na_2S_2O_4/Na_2SO_3$ was calculated according to Mayhew [24]. The absorption of the protein was monitored at 425 nm. The 100% absorption value was 0.19. The reaction mixture contained 100 mM HEPES/NaOH, 10 mM $MgCl_2$, 40 mM glucose; 1 $mg.ml^{-1}$ glucose oxidase; 5 mM $Na_2S_2O_4$, 1 mM Na_2SO_3, final pH = 7.0. [Av_2] = 22 μM, specific activity 1700 $nmoles.C_2H_2$ $reduced.min^{-1}.mgAv_2^{-1}$ and if present 5 mM MgATP or MgADP. O-O, Av_2 free; Δ-Δ, Av_2.MgATP; □-□, Av_2.MgADP. The solid line represents the n=2 curve. The calculated n-value through all data points is 2.2 ± 0.2.
The separate experiments give the following values.
Av_2 free: E_m = -389 mV, n = 2.1; Av_2.MgATP: E_m = -396 mV, n = 2.3;
Av_2.MgADP: E_m = -403 mV, n = 2.3.

Results of such experiments are shown in figure 2. As can be seen there are no significant differences between Av_2 in the presence or absence of adenine nucleotides. Calculated midpoint potential is -395 ± 5 mV and the slope is n = 2.2 ± 0.2. This result might explain that in the stopped flow experiments, no difference in redox behaviour of Av_2 in the presence or absence of adenine nucleotides was observed. However the results are in sharp contrast with the EPR redox titrations [20], in which the redox state of the protein was monitored by the intensity of its EPR signal. The significance of this difference will be discussed later (chapter VII, Discussion).

Fig. 3a shows the uptake of electron(s) by physiologically oxidized Av_2. In this method Av_2 is oxidized by a small amount of Av_1 under the conditions as described by Ljones and Burris [19].
After oxidation a non-saturating pulse of dithionite was added, Av_2 is rapidly partially reduced and then becomes reoxidized again. By extrapolation, the amount of Av_2 reduced by a known amount of dithionite can be calculated and therefore the number of electrons taken up.

However, several events make these type of experiments less conclusive, as shown in figure 3b. The reaction conditions are those of figure 3a except that the pH was 7.0 instead of 8.0 and the specific activity of Av_1 used was only 1100 nmoles C_2H_2 reduced.min^{-1}.mg Av_1^{-1} instead of 2000 nmoles C_2H_2 reduced.min^{-1}.mg Av_1^{-1}. It is clear that the oxidation is not fully reversible. The most likely explanation is that a loss of iron-sulphur cluster has occurred during the redox cycle. This is confirmed by the observation that excess dithionite results in a lower absorption at 425 nm than the originally reduced protein. Another factor that determines the outcome of an experiment is the rate at which oxidation takes place. The experiments have to be performed with highly active Av_1, otherwise a lag or slow oxidation after the non-saturating dithionite pulse occurred as shown in fig. 3b. This made the extrapolation less reliable. Another observation is that preparations of Av_2 of both high and low activity show the same absorption changes at 425 nm. It is also necessary that these experiments are performed at pH = 8.0, otherwise the redox potential of dithionite is not low enough to reduce Av_2 completely. But when all these pittfalls are circumvented, values of 1.9 ± 0.1 electrons/Av_2 are found (six experiments).

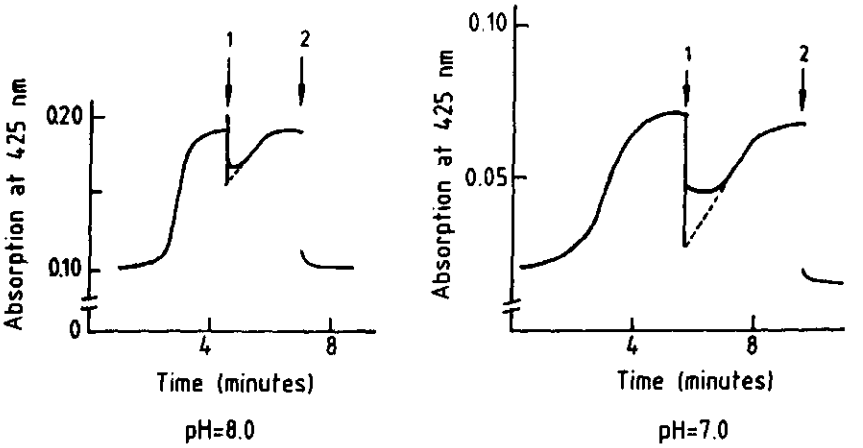

Figure 3A

Figure 3B

Figure 3A. Physiological oxidation of Av_2 and reduction by dithionite. The absorbance was monitored at 425 nm. The reaction mixture was as described in Materials and Methods plus 50 mM Tris HCl; 40 mM glucose; 1 mg.ml^{-1} glucose oxidase, [Av_2] = 29 μM [Av_1] = 0.30 μM, final pH as indicated. The specific activities of the proteins used were: Av_2: 1700 nmoles C_2H_2 reduced.min^{-1}.mgAv_2^{-1}; Av_1: 2000 nmoles C_2H_2 reduced.min^{-1}.mgAv_1^{-1}. At arrow (1) 10 μM $Na_2S_2O_4$ was added; at arrow (2) 5 mM $Na_2S_2O_4$.
Figure 3B. Specific activity of Av_2 as in fig. 3A; Av_1: 1100 nmoles.C_2H_2 reduced.min^{-1}.mgAv_1^{-1}. [Av_2] = 11 μM. At the time indicated by arrow (1) dithionite was added to a final concentration of 8 μM. At arrow (2) a saturating pulse of dithionite is given of 5 mM concentration final concentration.

In contrast to Ljones and Burris [19] we did not correct for inactive protein since we found that less active protein also gives colour changes.

From our electron counting experiments we conclude that physiologically or PMS oxidized Av_2 in the absence or the presence of MgATP can take up two electrons per molecule. The significance of the uptake of two electrons by Av_2 with respect to its catalytic function will be discussed in chapter VII (Discussion).

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CHAPTER IV.

Fully Active Fe-Protein of the Nitrogenase from *Azotobacter vinelandii* Contains at least Eight Iron Atoms and Eight Sulphide Atoms per Molecule

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The Fe-protein of the *Azotobacter vinelandii* nitrogenase enzyme complex contains a variable iron and sulphide content. The iron and sulphide content of the protein is dependent upon the specific activity. Up to a specific activity of 1000 nmol C_2H_4 produced · min^{-1} · mg Av_2^{-1}, three iron and three sulphide atoms per molecule Av_2 are found. At specific activities above 1000 nmol C_2H_4 produced · min^{-1} · mg Av_2^{-1}, a linear relationship between specific activity and iron and sulphide content of Av_2 is found. The maximum values found are 8.8 iron atoms and 8.6 sulphide atoms/molecule at a specific activity of 2250 nmol C_2H_4 produced · min^{-1} · mg Av_2^{-1}. Also the experimental molar absorption coefficients at 430 nm of the oxidized and reduced forms depend on the specific activity. The highest values found are 15.9 mM^{-1} cm^{-1} and 9.1 mM^{-1} cm^{-1}, respectively.

Since occasionally the preparations with specific activities around 3000 nmol · min^{-1} · mg^{-1} are isolated which contain more than 10 iron atoms and 11 sulphide atoms per molecule, it cannot be excluded that under certain physiological conditions Av_2 contains even more than two [4 Fe-4 S] clusters.

The addition of MgATP induces a conformational change in the Fe-protein which results in a higher reactivity with iron chelators. But irrespective of the specific activity, the amount of iron extracted from the protein after addition of MgATP never exceeds four atoms/molecule.

The results are discussed with respect to the present molecular model of the Fe-protein.

It is generally accepted that the Fe-protein of any nitrogenase enzyme complex has one [4 Fe-4 S] cluster [1 – 3]. This is based upon iron and sulphide determinations of various Fe-proteins of nitrogenase isolated from different bacteria like *Clostridium pasteurianum* [4 – 7], *Azotobacter chroococcum* [8, 9], *Klebsiella pneumoniae* [9, 10] and *Azotobacter vinelandii* [11]. Cluster extrusion experiments indicate that the iron in the Cp_2 preparations is quantitatively recovered as a similar product to that found with ferredoxin, i.e. a [4 Fe-4 S] cluster type [12].

The Fe-protein from *C. pasteurianum* behaves like a one electron donor/acceptor upon redox titrations in EPR experiments [13]. Also colorimetric measurements on Cp_2 at 430 nm indicate a one-electron behaviour upon oxidation/reduction in the physiological reaction [14]. If one takes into account that a [4 Fe-4 S] cluster is expected to transfer one electron [15], then it is not surprising that component II is considered to be a one-electron donor with one [4 Fe-4 S] cluster.

However, there are reports that the Fe-protein from *Azotobacter* can accommodate two electrons [16, 17]. Thorneley et al. [17] showed that oxidized Ac_2 can take up two electrons but they suggested that the second electron, taken up slowly, is due to inactive protein. Braaksma et al. [16] demonstrated that Av_2 with or without MgATP behaves as a two-electron donor in a redox titration followed by EPR spectroscopy. Lowe [18] proposed from the anisotropic linewidth and low integrated intensities of the EPR signal of component II of nitrogenase that a second rapidly relaxing paramagnetic center is present in the protein.

In this article we present data that highly active Av_2 contains more than four functional iron and sulphide atoms thus explaining the previous observation [16] that Av_2 can act as a two-electron carrier.

MATERIALS AND METHODS

Isolation of the nitrogenase proteins and standard nitrogenase activity assays were run as described earlier [16] in a mixture containing 50 mM Hepes/KOH, 10 mM ATP, 15 mM $MgCl_2$, 10 mM creatine phosphate, 0.3 mg creatine kinase/ml, 1 mg bovine serum albumin/ml, final pH = 7.5.

Assay mixtures and solutions were made anaerobic by evacuating and filling with argon plus additional flushing with argon to remove the last traces of oxygen. Dithionite was added from a stock solution (200 mM, 25 mM Tris/HCl, final pH = 7.5), prepared fresh daily, to a final concentration of 20 mM. Acetylene was added to a final concentration of 20 %.

Av_1 and Av_2 were isolated as described earlier [16]. Av_2 purified by this method has a specific activity between 1500 – 1800 nmol C_2H_4 min^{-1} · mg AV_2^{-1}. Protein with a lower specific activity was obtained by exposing Av_2 for 2 min to air. To produce highly active Av_2 an additional concentration step was necessary, even after storage of highly active protein in liquid nitrogen. Av_2 was concentrated on a small DEAE-cellulose (DE-32) column (1 × 5 cm) and was eluted with 90 mM $MgCl_2$. This resulted in an increase in specific activity up to 2250 nmol C_2H_4 produced · min^{-1} · mg Av_2^{-1}. A Bio-Gel P-6DG (Bio-Rad) column (1 × 5 cm) was used to desalt and to exchange buffers. When not indicated the column was equilibrated with 25 mM Hepes (pH = 7.4), 5 mM $MgCl_2$. To maintain the activity of Av_2 around 2200 nmol C_2H_4 formed

Abbreviations. The Mo-Fe and Fe protein of the nitrogenase of *Azotobacter vinelandii*, *Azotobacter chroococcum*, *Clostridium pasteurianum* and *Klebsiella pneumoniae* are referred to as Av_1 and Av_2, Ac_1 and Ac_2, Cp_1 and Cp_2 and Kp_1 and Kp_2 respectively; EPR, electron paramagnetic resonance; BPS, bathophenanthroline disulfonate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid.

$\cdot min^{-1} \cdot mg\ Av_2^{-1}$, it was necessary to run the molecular sieve column in the presence of $MgCl_2$. In the absence of $MgCl_2$ the activity dropped to 1800 nmol C_2H_4 formed $\cdot min^{-1} \cdot mg\ Av_2^{-1}$.

The specific activities of the Av_1 enzyme preparations were 2400 – 3000 nmol C_2H_2 produced $\cdot min^{-1} \cdot mg\ Av_1^{-1}$ and of Av_2 preparations 1500 – 2250 nmol C_2H_4 produced $\cdot min^{-1} \cdot mg\ Av_2^{-1}$. The purified Av_1 and Av_2 preparations were pure as judged by one-dimensional and two-dimensional SDS/polyacrylamide gel electrophoresis. Protein concentrations were routinely estimated with the method of Sedmak and Grossberg [19]. Other protein determination methods, like the biuret method [20] and the Lowry method [21], were performed on purified Av_1 and Av_2, as well as dry weight measurements upon Av_2. Dry weight determinations showed that the protein determination of purified Av_2 by the method of Sedmak and Grossberg [19] gave an underestimation of 25 – 30%. Protein concentrations of Av_2 measured with the method of Sedmak and Grossberg [19] were corrected for the underestimation. No correction factor is necessary for protein determinations with the biuret or the Lowry methods. For all protein determinations bovine serum albumin was used as a standard. The bovine serum albumin concentration was determined by the absorption at 279 nm, $\varepsilon = 44.67\ mM^{-1} \cdot cm^{-1}$.

Treatment with Chelex-100 (Bio-Rad) was performed batchwise. An assay bottle (7.0-ml stoppered with a suba seal) with 0.1 g of dry Chelex-100 was evacuated and filled with argon three times plus an additional 5-min flushing to remove the last traces of oxygen. 0.2 ml of anaerobic buffer solution was added and again evacuated and filled with argon. Dithionite was added to a final concentration of 2 mM. After a 15-min equilibration, 0.2 ml of Av_2-containing solution was added. During the 10-min incubation time the bottle was gently shaken. The Chelex-100 was allowed to settle and the supernatant was separated from the Chelex by a syringe, with a thin needle to prevent Chelex-100 contamination.

It was checked that this procedure removes extra added iron efficiently. Iron was routinely extracted from Av_2 by adding of an aliquot of the enzyme solution to 30% trichloroacetic acid giving a final concentration of 20%, followed by dilution with water to a 5% concentration. The denaturated protein was centrifuged and the iron content of the supernatant was determined by the method of Massey [22] in quadruple with bathophenanthroline disulfonate (BPS) as iron chelator instead of *o*-phenathroline disulfonate. The reliability of the iron determination was 8%. Atomic absorption was done with an Instrumentation Laboratory aa/ae spectrophotometer 357 upon the supernatant of the trichloroacetic acid treatment.

The sulphide concentration was estimated according to Fogo and Popowski [23] as modified by Brumby et al. [24]. It was necessary to measure at low protein concentrations due to quenching of the colour development by the protein. Determinations done in quadruple with less than 150 µg Av_2 added per 1.7-ml assay volume, gave a linear relationship between the amount of protein added and colour development. The reliability of the sulphide determination was 10%.

The molar absorption coefficient obtained with a calibration curve was the same as the value reported by Brumby et al. [24]. It was checked that the elution buffer used did not make any contribution to the colour development of the sulphide assay, by adding quantities comparable to the enzyme solution.

Oxidation of Av_2 was performed on a Bio-Gel P-6DG column by running Av_2 through a layer of phenazine methosulfate and subsequent desalting as described by Yates et al. [25]. Enzymic oxidation was achieved by trace amounts of Av_1 in the presence of an ATP-regenerating system. The molecular mass of Av_2 was taken as 63 kDa for the dimer, as determined by amino acid analysis [26, 27].

Absorption measurements were performed at 430 nm on either an Aminco-DW2A or a Zeiss spectrophotometer type M4Q III, PI-2. Phenazine methosulfate and BPS were obtained from Sigma. Mohr's salt and Na_2S were obtained from Merck. Na_2S was calibrated iodometrically according to [28].

All other chemicals were from highest purity commercially obtainable.

RESULTS

We used proton-induced X-ray emission spectroscopy (PIXES) to detect whether other transition metals besides iron are present in Av_2. An unexpectedly high level of iron and low amounts of copper and zinc were detected. The copper content proved to be variable between 0.3 and 1.3 atom/molecule Av_2. Since there was no correlation with the specific activity of the protein preparation, we concluded that copper is not essential.

The Fe-standard curve determined by the modified colorimetric method of Massey (see Methods and Materials) gave a molar absorption coefficient of $21.67 \pm 1.60\ mM^{-1} \cdot cm^{-1}$ at pH = 7.4 and $20.98 \pm 0.04\ mM^{-1} \cdot cm^{-1}$ at pH = 4, values almost equal to those given by Blair and Dient [29], who described the iron-chelating reaction with BPS. It was confirmed that the pH of the incubation mixtures was 4 after the addition of saturated ammonium acetate as recommended by Massey [22].

Extraction of the iron in Av_2 is not effective in 5% trichloroacetic acid, especially with protein of a high specific activity. But an initial incubation of Av_2 in 20% trichloroacetic acid (final concentration) and a subsequent dilution to 5% results in an immediate release of this tightly bound iron. A comparison of the results obtained by this method with those obtained by atomic absorption on different samples indicate that the iron content of Av_2 determined by the colorimetric method gives an underestimation of 20 ± 10%. This is not unusual; a similar situation has been described by Yates and Planqué [8]. Determination of the iron content of Av_1 by the colourimetric method described in Materials and Methods yielded 28 iron atoms/molecule Av_1, which is in accordance with the literature [1, 3].

The sulphide analysis was performed with two methods. The method of Chen and Mortenson [30] was used, because we expected a similar quenching by the dithionite oxidation products as they described. The calibration curve for this method gave the same molar absorption coefficient as was given by Chen and Mortenson. However, dithionite-free Av_2 also gave a considerable quenching as was the case with the method of Brumby et al. [24]. We therefore used low protein concentrations for the determination (see Materials and Methods). Both methods gave similar results but for convenience we used the method of Brumby et al. [24].

Fig. 1 shows the relation between iron content from a large number of different preparations of Av_2 and their specific activities. It is clear that only above a specific activity of 1000 nmol C_2H_4 produced $min^{-1} \cdot mg\ Av_2^{-1}$ is an increase in iron content of Av_2 observed.

The sulphide determinations follow a similar pattern as can be derived from Fig. 2. It is clear from this figure that the average ratio of iron to sulphide in different preparations is close to one. This means that an increase in iron content is associated with an increase in sulphide content, thus in [Fe-S]

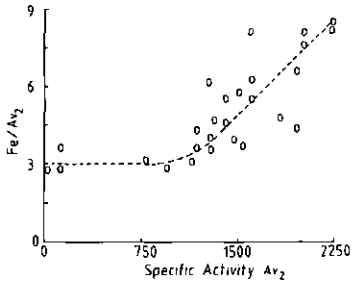

Fig. 1. *Relation between the number of iron atoms per molecule Av_2 and the specific activity of different Av_2 preparations prepared as described in Materials and Methods.* Iron was determined colorimetrically as described in Materials and Methods; specific activity was measured as described in Materials and Methods and is expressed as nmol C_2H_4 produced · min^{-1} · mg AV_2^{-1}. All data points represent different preparations. The dotted line is a visual fit through the data points

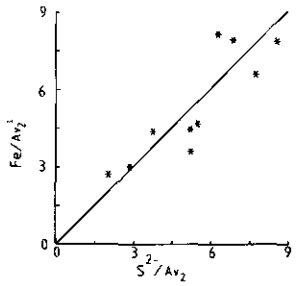

Fig. 2. *Relation between the number of iron and sulphide atoms per molecule Av_2 of several different preparations with different specific activities.* Each plotted point is the result of an iron and sulphide determination of one Av_2 preparation. The plotted line gives the expected ratio Fe/S = 1

Table 1. *A comparison of several treatments on Av_2 to estimate total iron contents and non-specifically bound iron*
The Chelex-100 treatment and the molecular sieve column (Bio-Gel P-6DG) were performed as described in Materials and Methods

Treatment	Specific activity		Fe/Av_2	S^{2-}/Av_2
	before the treatment	after the treatment		
	nmol · min^{-1} · mg^{-1}		atoms/molecule	
As isolated	2152	–	8.6	6.2
Molecular sieve column	2159	2159	8.0	6.8
Chelex-100 and molecular sieve column	2135	2110	7.6	7.3

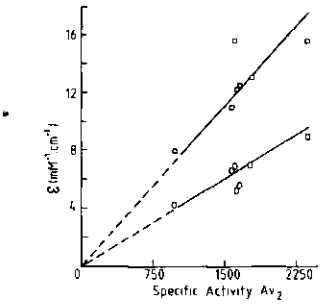

Fig. 3. *Relation between molar absorbance of oxidized (□) and reduced (○) Av_2 at 430 nm of several preparations with different specific activities prepared as described in Materials and Methods.* The lines drawn are visual fits through the data points. For clarity the extrapolation to zero was made (dotted parts of the lines). The specific activity of Av_2 was measured and oxidation was performed as described in Materials and Methods. Specific activity of Av_1 is expected as nmol C_2H_4 produced · min^{-1} · mg Av_2^{-1}. Molar absorbance, ε, was measured at 430 nm

cluster content of the protein. After almost complete inactivation there are still about three iron and three sulphide atoms per molecule Av_2 left.

An estimation of the non-specifically bound iron was made by a Chelex-100 treatment of Av_2. As can be seen in Table 1, this treatment hardly affects the specific activity and does not remove iron.

The higher amount of iron is also clear from the absorption at 430 nm of the oxidized and reduced forms of Av_2 (Fig. 3). As seen from Fig. 3, above a specific activity of 1000 nmol C_2H_4 produced · min^{-1} · mg Av_2^{-1} there is also linear relationship between specific activity and molar absorbance of the oxidized and reduced protein. At a specific activity of 2250 nmol C_2H_4 formed · min^{-1} mg Av_2^{-1} the molar absorbance of the protein oxidized enzymically or with phenozine methosulfate is 15.9 $mM^{-1} \cdot cm^{-1}$ and of the dithionite-reduced protein 9.1 $mM^{-1} \cdot cm^{-1}$. This protein contains about 8 Fe atoms and 8 S^{2-} atoms/molecule Av_2. The molar absorbances and the iron and sulphur content of Av_2 are twice as high as the values reported for Ac_2 [8].

Due to their lability, the specific activity of the Fe-proteins has always been a matter of concern. Only Cp_2 can be isolated in a more active form [31], 2700 nmol C_2H_4 produced · min^{-1} · mg Cp_2^{-1}. Nowadays a specific activity of 2000 nmol C_2H_4 produced · min^{-1} mg^{-1} for Av_2 [11, 17, 32] and Ac_2 [8, 9] is not unusual. We found that highly active Av_2 could be reproducibly obtained by a concentration step as described in Materials and Methods. With starting material of specific activity 1500–1800 nmol C_2H_4 produced · min^{-1} · mg Av_2^{-1}, this method yields preparations with specific activities around 2250 nmol C_2H_4 produced · min^{-1} mg Av_2^{-1}. Preparations with this specific activity contain 8.8 Fe/atoms and 8.6 S^{2-} atoms/molecule Av_2. However, upon freezing in liquid nitrogen and thawing under argon the activity drops immediately to 1760 nmol C_2H_4 produced · min^{-1} · mg Av_2^{-1}. The sulphide content is unaffected, but the iron content declines from 8.8 to 4.6 atoms/molecule Av_2. This could be due to the presumed volatility of iron chloride [33] but control experiments with a reduced iron chloride solution did not indicate any loss of iron upon subsequently freezing, thawing, evacuating and flushing

with argon. So we assume that the iron is somehow very tightly bound to (inactive) protein and is not released by 20% trichloroacetic acid. This was confirmed by treating the enzyme solution by strong acid and wet ashing as recommended by Van de Bogart and Beinert [33]. In Av_2 slutions with low ratios of iron to sulphide, after freezing and thawing the protein, wet ashing proved to be necessary to determine the total iron content accurately. Sometimes Av_2 isolations yielded Av_2 protein with specific activities around 3000 nmol C_2H_4 produced · min $^{-1}$ · mg $Av_2{}^{-1}$. These particular preparations contained 10.6 iron and 11.3 sulphide atoms/molecule Av_2. However, this type of preparation lost 30% of its activity upon passage over a molecular sieve or freezing in liquid nitrogen followed by subsequently thawing under argon.

The active Fe-protein of nitrogenase shows a slow reaction with iron chelators. Walker and Mortenson [6] found that addition of MgATP increases the rate of the iron-chelating reaction. This feature was also observed by Ljones and Burris [14] with the iron-chelator BPS. They also found upon addition of MgATP a large increase in rate and extent of the iron-chelating reaction. MgATP is thought to induce a conformational change in the Fe-protein which results in a higher accessibility of iron chelators to the iron atoms in the [Fe-S] cluster. So the increase in rate upon addition of MgATP is ascribed to active protein, whereas the initial increase of iron chelating before the addition of MgATP is thought to come from inactive protein. Ljones and Burris [14] and Hageman et al. [32] used this method to calculate the percentage active protein of a given preparation. As shown in Fig. 4 the addition of MgATP gave irrespective of the specific activity, a complex of three iron atoms with BPS/molecule Av_2. As can be seen from our results, calculation of the percentage active protein by dividing the amount of iron chelated before and after the addition of MgATP gives erratic results. It is also clear that not all the iron is extracted from Av_2 by BPS in the presence of MgATP.

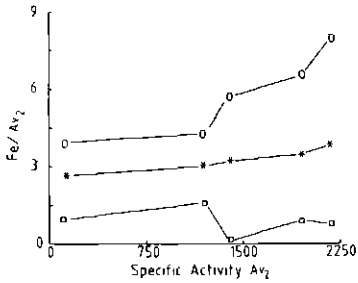

Fig. 4. *Relation between the specific activity of Av_2 and the iron content of Av_2 as measured with different methods.* The amount of iron was detected with the reaction of the iron chelator BPS. (□—·· □) Av_2 as isolated; (× — ×) Av_2 as isolated with 5 mM MgATP present, chelation was completed within 5 min; (○ ··○) total iron content of Av_2 after extraction with trichloroacetic acid. Specific activity is expressed as nmol C_2H_4 produced · min $^{-1}$ · mg $Av_2{}^{-1}$. Iron content is expressed as iron atoms/molecule Av_2. Iron and the specific activity of Av_2 were measured as described in Materials and Methods

DISCUSSION

The specific activity of an Fe-protein has always been a matter of concern. In the early isolations specific activities for Av_2 of around 1500 nmol C_2H_4 produced · min $^{-1}$ mg $^{-1}$ were usual. Iron determinations on those preparations never yielded more than four iron atoms per molecule and mostly less. As can be seen in Fig. 1, there is a considerable scattering in the number of iron atoms found per molecule. It is therefore important to measure a considerable number of preparations to be sure that Fe-protein preparations with a high specific activity have a higher iron content. Besides the specific activity of the protein, another factor was found to be important for the determination of iron content of Av_2. Not all iron might be released during a relatively mild denaturation, such as cluster extraction [12] or the iron-chelating reaction with BPS after the addition of MgATP [34].

Since we cannot judge from the literature that all these effects have been recognized, it is difficult to explain the lower values found for the Fe-proteins. For some reports we can give an indication why a relatively low amount of iron is found. For instance, Kp_2 was analysed for iron and sulphide content by Thorneley [9] and Eady et al. [10]. But we consider the specific activity to be too low for a protein containing more than four iron atoms per molecule. Ac_2 was analysed by Yates and Planqué [8]. The specific activity of the Ac_2 preparation was high enough to yield more than the four iron and sulphide atoms found by the authors. The molar absorbance of the oxidized and reduced forms of Ac_2 at 430 nm as reported [8], however, indicates a low iron-sulphide content compared with our preparations (see Fig. 3).

Av_2 as investigated by Burgess et al. [11] contains four iron atoms/molecule. The specific activity of 2000 nmol · min $^{-1}$ · mg $^{-1}$ was measured by hydrogen evolution which gives an overestimation compared with C_2H_4 production. It was not clear whether the four samples analysed for Fe had the high specific activity mentioned in the article. Unfortunately no sulphide analyses were done on these samples.

Cp_2 was thoroughly investigated by several groups [4, 12, 31]. The specific activity seems high enough in the case of Moustafa and Mortenson [31]. But they could only detect two iron atoms per molecule Cp_2 or, corrected for the molecular mass of 40 kDa which they use, 2.8 iron atoms/molecule. The sulphide content was of the same magnitude. This low number of iron atoms, which can hardly account for even one [4 Fe-4 S] cluster, can be attributed, in our view, to the scatter in iron and sulphide determinations (see Fig. 1) and possibly the given specific activity of 2700 nmol C_2H_4 produced · min $^{-1}$ mg $Cp_2{}^{-1}$ is over-estimated by using a cell-free extract without Cp_2 activity to determine the activity of the Cp_2 preparations, as was shown by Eady et al. [7].

Later Nakos and Mortenson [4] reported that the number of iron and sulphide atoms present in a molecule of Cp_2 is not 2.8 but 4.0. But unfortunately they did not mention their specific activities, although the isolation method applied can give rise to good preparations. High concentrations of trichloroacetic acid as routinely used by us, did not always extract all the iron, especially when the protein had been frozen in liquid nitrogen and thawed under argon. For this kind of protein the degradation method of Van de Bogart and Beinert [33] and Lovenberg et al. [35] proved to extract equal amounts of iron and sulphide indicating that probably all the iron was extracted.

To accommodate two [4 Fe-4 S] clusters in Av_2 a minimal number of eight cysteine residues per dimer is necessary, so that all the iron atoms of the two clusters are connected with a

cysteinyl side chain. From the amino acid composition of Av_2 [27] it is known that there are 14 cysteines per dimer. If in Av_2, as in Cp_2 [3], four of the cysteines react with 5,5'-dithiobis(2-nitrobenzoic acid) without any loss of activity, there are still enough cysteines available to bind these two [4 Fe-4 S] clusters. There is even a possibility that in this model a third cluster, being very labile, can be accommodated in Av_2, thus explaining our occasional high activity related with more than 10 iron and 11 acid-labile sulphur atoms per molecule Av_2.

The molar absorbance difference of an oxidized and reduced [4 Fe-4 S] cluster in a ferredoxin is $\Delta\varepsilon_{430}$ nm (ox-red) $= 7.0\,mM^{-1}\cdot cm^{-1}$ [36]. We found for Av_2 preparations with specific activities around 2250 nmol min^{-1} mg^{-1} and with eight iron atoms and eight sulphide atoms per molecule of Av_2 a value of $\Delta\varepsilon_{430}$ (ox-red) $= 6.8\,mM^{-1}\cdot cm^{-1}$. This could mean that only one of the two [4 Fe-4 S] clusters undergoes redox changes in the potential spanned by phenazine methosulfate ($E_h \approx +80$ mV) and dithonite ($E_h \approx -500$ mV). The redox state of the second [4 Fe-4 S] cluster is not influenced by the applied redox span. If this possibility is true, one cannot expect an extrapolation of the molar absorbance of the protein to zero when the amount of iron and sulphide per molecule of Av_2 diminishes (see Fig. 1 and 3). In this case one expects a value of the molar absorbance above zero, the value of the Fe-S cluster that is not influenced by the applied redox span. As can be seen from Fig. 3, this is not the case. Another observation that argues against this possibility is that the redox changes of Av_2 when measured by the intensity of the EPR signal of Av_2 is associated with a two-electron redox reaction [16]. A ferredoxin type of [4 Fe-4 S] cluster can accommodate only one electron in its two redox states (EPR-visible and EPR-silent).

The second possibility that the molar absorbance of the [4 Fe-4 S] clusters of nitrogenase component II has quite different spectroscopic properties as compared with the [4 Fe-4 S] clusters present in ferredoxins is more likely. Low molar absorbance values for nitrogenase component II are reported in the literature. Yates and Planqué [8] reported for Ac_2 $\Delta\varepsilon_{430}$ (ox-red) $= 1.85\,mM^{-1}\cdot cm^{-1}$ and Ljones and Burris [14] for Cp_2 $\Delta\varepsilon_{340}$ (ox-red) $= 3.8\,mM^{-1}\cdot cm^{-1}$. Unfortunately there are no indications in the literature that in these cases specific activity measurements iron, and sulphide determinations and spectra of the oxidized and reduced proteins were performed on the same samples, as in our case.

There is experimental evidence that MgATP interacts with the Fe-protein followed by detectable changes in physical and chemical properties of the protein. For instance, addition of MgATP results in a higher accessibility of iron atoms to iron chelators [5, 6]. This is explained by assuming a conformational change upon binding of MgATP which results in an exposure of the presumed [4 Fe-4 S] cluster. Fig. 4 shows that the explanation of Ljones and Burris [14] ascribing the initial iron-chelating reaction to inactive protein which is no longer able to protect its [4 Fe-4 S] cluster, while the increase an iron-chelation rate upon addition of MgATP is ascribed to active protein, is not correct.

The cluster exposed after MgATP addition is different from the cluster seen with EPR spectroscopy. Even almost completely inactive protein, which shows no EPR signal, still has three or four iron (and sulphide) atoms left and still shows the exposure of Fe upon binding of MgATP.

In this light it can be stated that the stoichiometry of active nitrogenase proteins Av_2 and Av_1 as has recently been proposed by Wherland et al. [37] is questionable in the light of the data presented here. Only with preparations characterized in terms of specific activities, iron and sulphide contents can meaningful data be expected with nitrogenase preparations from any source.

A second paramagnetic cluster was predicted by Lowe [18], who deduced from EPR spectroscopy on Kp_2 that there must be a second paramagnetic centre close to and with a strong interaction with the EPR-visible [4 Fe-4 S] cluster. Recent unpublished results of our group clearly indicate that a fast-relaxing species is present in highly active preparations which can be observed at low temperature (4 K) and relatively high microwave power (about 12.5 mW). We explain our data by assuming the presence of another [4 Fe-4 S] cluster. One cluster is the EPR-visible cluster, the second is a fast-relaxing one. The normal and fast-relaxing cluster are most probably coupled in the way Lowe [18] proposed.

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Chapter V. THE IRON SULPHIDE CONTENT OF Fe-PROTEIN OF NITROGENASE FROM AZOTOBACTER VINELANDII IS DETERMINED BY THE GROWTH CONDITIONS OF THE CELLS

Summary

The iron and acid labile sulfur contents and stability of isolated Fe-protein of nitrogenase from Azotobacter vinelandii is determined by growth conditions of the cells.
Fe-protein isolated from cells grown at a low free oxygen concentration contains 3.5-3.8 iron and acid labile sulfur atoms. The protein is relatively stable. When Fe-protein was isolated either from cells treated with levels of oxygen that inhibited growth or from cells grown at a high free oxygen concentration (>30 μM), the Fe-protein had iron and sulphide contents above 4, but the protein was much more labile than regular Fe-protein which was reflected in loss of activity, iron and sulphide atoms.

Since the whole cell nitrogenase activity was not inhibited by an oxygen shock we therefore suggest that dioxygen alters Fe-protein so that its activity in vivo is not affected, but in vitro the altered protein is more labile.

Introduction

Nitrogenase consists of two easily separable proteins. One protein is called Fe-protein and the other MoFe-protein. It is generally assumed that Fe-protein contains one [4Fe-4S] cluster [1-3]. This is based upon iron and sulphide determinations of various Fe-proteins isolated from different bacteria like C.pasteurianum [4-7], A.chroococcum [8,9], K.pneumoniae [9,10] and A.vinelandii [11]. Cluster extrusion experiments indicated that the iron atoms in Cp_2 preparations can be quantitatively recovered as a product similar to that extracted from ferredoxin, i.e. a [4Fe-4S] cluster [12]. Recently we reported that Fe-protein could be isolated from A.vinelandii with more than 4Fe and $4S^{2-}$ atoms per molecule [13].
In this article we show that the growth conditions of the cells determine the Fe and S^{2-} contents of the Fe-protein from A.vinelandii.

Materials and Methods

Growth conditions of the cells

Azotobacter vinelandii ATCC 478 was grown in a batch culture of 20 liters in a Bio-Engineering fermentor type LP30. The medium was as described by Newton et al. [14], temperature was 30°C. Fermentation was started by inoculating 20 liters of medium with approximately 300 mg bacterial protein. The air input was set at 15 liter.min^{-1} and the stirring speed at 80 rpm. During the first 12 hours the oxygen concentration decreased and the culture growth became oxygen-limited. Generation times are 4-5 hours in this stage of the growth. The nitrogenase activity of the whole cells is around 50 nmoles C_2H_2 reduced.min^{-1}.mg protein^{-1}. After 15 hours of growth the aeration was raised by increasing the stirring speed to 160 rpm. After a short adaptation (less than 1 hr) the culture started to grow logarithmically with a generation time of about 3 hours. These cells have a whole cell nitrogenase activity around 90 nmoles C_2H_2 reduced.min^{-1}.mg protein^{-1}. The free-oxygen concentration which was enhanced at the onset of the increase in aeration dropped within 90 minutes to almost zero. At a protein concentration of 0.3 mg.ml^{-1} an oxygen shock was given. The aeration was increased to 30 liters air.min^{-1} and the stirring speed to 450 rpm. The measured free oxygen concentration increased to 100% saturation. The respiration capacity of the culture was not high enough to lower the free oxygen concentration within a very short time and both nitrogen fixation and growth were inhibited. Within two hours the cells adapted their respiration rate and the free oxygen concentration dropped and at about 30% saturation the cells started to grow again.

At different times, samples were taken from the fermentor and the in vivo nitrogenase activity was measured under optimal conditions for nitrogen fixation as described earlier [15]. Cells were harvested by centrifugation at different times for the isolation of Fe-protein. The harvesting took less than 1 hour during which the culture was not aerated. Standard nitrogenase activity assays and sulphide determinations were performed as described earlier [13].

Isolation procedures

The isolation of Fe-protein is a modification of the purification method described earlier [13]. A typical purification used approximately 50 g wet weight of cells. Cells were disrupted with a Manton-Gaulin homogenizer type 15 M (Gaulin Corporation, Everett, MA, USA) in 100 ml of 100 mM Tris-HCl pH 8.0; 5 mM $MgCl_2$; 0.01 mg/ml DNase; 0.01 mg/l RNase. The cell-free extract was obtained by centrifugation (20,000 g; 20 minutes). The specific activity varied between 30-80 nmoles C_2H_2 reduced.min^{-1}.mg $protein^{-1}$ depending upon the nitrogenase activity of the whole cells. After this stage, all buffers were flushed with argon and contained 2 mM dithionite. The cell-free extract (approximately 4 g of protein) was loaded onto a DEAE-cellulose column (2.5 x 10 cm), which was equilibrated with 50 mM Tris-HCl, pH 7.4. The column was subsequently washed with the equilibration buffer and with equilibration buffer containing 80 mM NaCl. An orange/red fraction containing nitrogenase protective protein [16] was eluted in this stage.

Then a gradient of 160 ml of 100-550 mM NaCl in equilibration buffer was applied, crude Av_1 eluted at 150-200 mM NaCl, crude Av_2 (with flavodoxin) eluted at 200-300 mM NaCl. Crude Av_2 was concentrated on a small DEAE column (1 x 2 cm). The next step was gel filtration of Av_2 over a molecular sieve column (Sephacryl S-200).

Av_2 was detected by its absorption at 425 nm. The elution profile was a Gaussian curve. The first fractions had a low specific activity and sometimes contained traces of Av_1. Most of the protein in these fractions was Av_2 as judged by SDS polyacrylamide electrophoresis. The activity of these fractions was low. The most concentrated fractions had the highest specific activity. These fractions were frozen by dropping the solution in liquid nitrogen and stored. If necessary, Av_2 was concentrated by loading onto a small DEAE column (1 x 2 cm), extensively washed with 25 mM HEPES, pH 7.4, 5 mM $MgCl_2$ and 2 mM dithionite, and eluted with 90 mM $MgCl_2$ in the same buffer. In this way all non-specifically bound iron, if present, was removed. Since denatured protein remained on the column, this step sometimes results in an increase in specific activity. If necessary, protein was desalted over a Biogel P6DG column (1 x 5 cm).

Analytical methods

Sulphide determinations were performed anaerobically under argon as recommended by Beinert [17]. Iron was analyzed by the wet washing method [18] and by the method as described by Lovenberg *et al*. [19]. We slightly modified the last method. After 10 minutes at 80°C in 1% hydrochloric acid, the protein solution was not homogeneous. By whirl-mixing, the denatured protein was resuspended and a homogeneous sample was taken. After the pH of the sample was brought to 4-4.5 with ammonium acetate, SDS was added to a final concentration of 1% to keep denatured protein into solution. The assay was performed as described earlier [13]. The ascorbic acid needed in this assay was prepared anaerobically. Protein concentrations were estimated by the microbiuret method [20].

Results

In figure 1 a growth curve of a batch culture of *Azotobacter vinelandii* is shown. The free oxygen concentration, which is expressed as percentage of air saturation in the culture medium, is the result of air input minus the respiration of the cells. Cultures were harvested at different times.

- Cells, grown logarithmically at a low free oxygen concentration, were harvested approximately 20 hours after inoculation. These cells are considered as regularly grown cells (type 1).
- Cells were harvested immediately after an oxygen-shock as described in Materials and Methods (type 2).
- Cells were harvested after the cells had regained logarithmic growth after an oxygen shock (type 3).

From these three types of cells Fe-protein was isolated. The yield of Fe protein and the specific activities are given in Table 1. It can be seen that the specific activity and the yield of the Fe-protein isolated from a logarithmically grown culture (type 1) are high and that the iron and sulphide content of the protein are somewhat below 4 atoms per molecule. The Fe-protein preparation thus obtained is relatively stable; freezing and thawing or concentrating on a small DEAE cellulose column does not affect the specific activity dramatically.

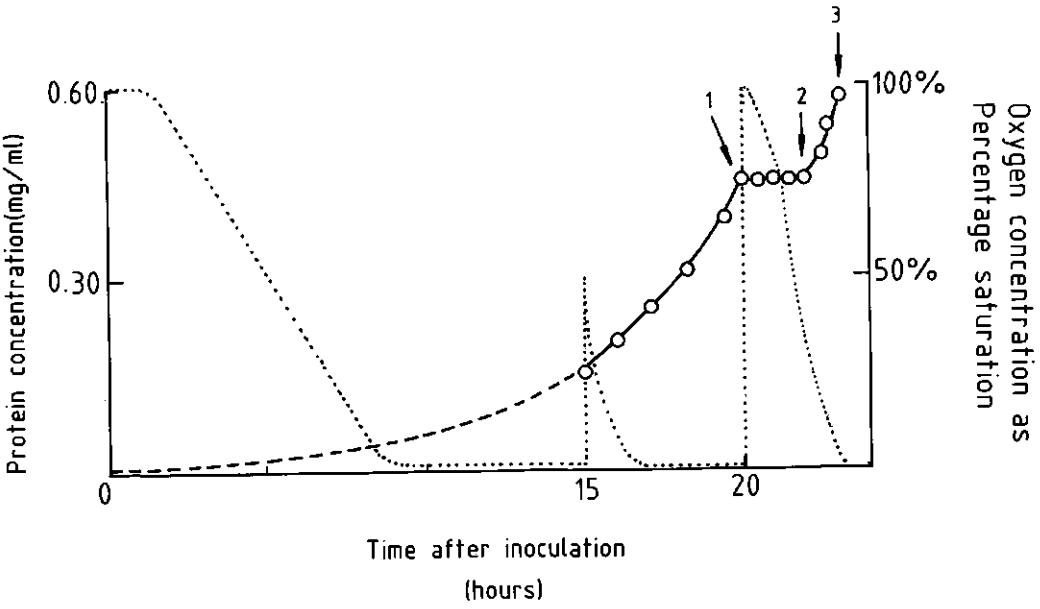

Figure 1. Growth curve of an A.vinelandii culture. Fermentation as described in Materials and Methods. The fermentor was inoculated with 280 mg bacterial protein. Air input was 15 $l.min^{-1}$, stirring speed was 80 rpm. 15 Hours after inoculation the aeration was increased (stirring speed 120 rpm). After 21 hours, an oxygen shock was given (air input 30 $l.min^{-1}$; stirring speed 450 rpm). At the indicated times (arrow 1, 2 and 3 the different types of cells were harvested.
o-o, protein concentration; ...., O_2 concentration. The arrows 1, 2 and 3 indicate the moment of harvest of respectively cells type 1, 2 and 3.

It was not possible to obtain highly active Fe-protein from cells harvested at the end of the oxygen-shock, before growth had started (type 2). The recovery of activity during purification was low (20%) and a large fraction of nitrogenase protein without any activity was eluted from the first DEAE-column at a salt concentration of 200 mM NaCl between fractions with MoFe-protein and Fe-protein activity. Further handling of these Fe-protein preparations resulted in a further loss of activity. Fe-protein isolated from cells that had regained logarithmic growth after an oxygen shock (type 3 cells), had a specific activity about equal to that isolated from type 1 cells. However, the iron and sulphide contents of the Fe-protein were above 4 (table 1). These values were measured with protein preparations immediately after elution from the molecular sieve column (the last purifica-

tion step). When the protein purified this way was stored in liquid nitrogen and after thawing concentrated on a small DEAE-cellulose column the specific activity dropped to about 1700 and the iron value declined to 3.4, although the sulphide content was still high (5.0). Further handling (chromatography, freezing/thawing) of the protein did not affect both the iron content and the activity, but the sulphide content dropped to the same value as that of the iron content.

Since preservation of activity during a purification might be an indication of the stability of a protein, we studied the effect of time on the activity of Fe-protein isolated from type 1 and type 3 cells (fig. 2).

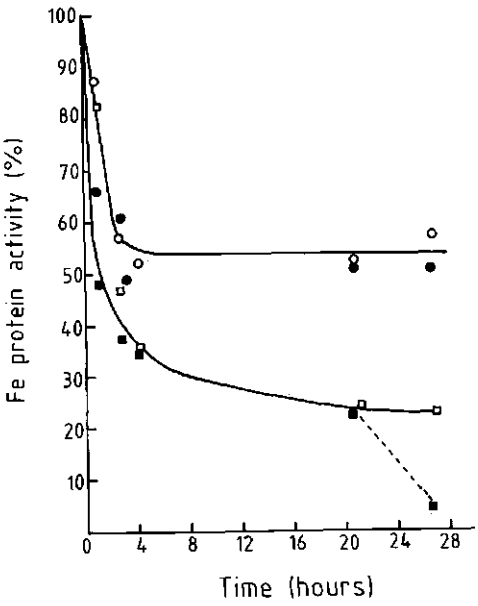

Figure 2. Stability of Fe-protein isolated from regularly grown cells (type 1) and from oxygen shocked cells (type 3).
Fe-protein type 1 cells: 100% activity = 1650 nmoles C_2H_2 reduced.min^{-1}.mg Fe $protein^{-1}$ (after freezing and thawing).
Incubation conditions: 25 mM TES-NaOH, pH = 7.4, 2 mM dithionite and 53 mM NaCl.
●-● 12.8 µM Fe-protein, 10 µM BSA; o-o 12.8 µM Fe-protein, 10 µM MoFe-protein.
Fe-protein from type 3 cells: 100% activity = 1750 nmoles C_2H_2 reduced.min^{-1}.mg Fe-$protein^{-1}$ (after freezing and thawing); incubation conditions: 2 mM dithionite and 36 mM NaCl.
■-■; 10µM Fe-protein, 10 µM BSA; □-□ 10 µM Fe-protein, 10 µM MoFe-protein.
Mo-Fe-protein activity was in both cases 1500 nmoles C_2H_2 reduced min^{-1}.mg MoFe-$protein^{-1}$.

Prolonged storage at room temperature with a slight argon overpressure, affects the Fe-protein isolated from oxygen shocked cells (type 3) more than from regular grown cells (type 1).
A semi-logarithmic plot of these data does not yield a straight line (not shown) thus the process of inactivation is complex and could be ascribed to a number of different structural changes in the protein. Remarkable is the stabilizing effect of the MoFe-protein at the end of the inactivation process of the Fe-protein isolated from oxygen shocked cells (type 3 cells) (observed in two experiments).

To get more information about what happens with the activity of Fe-protein *in vivo* during an oxygen shock, we studied the whole cell activity during an oxygen shock (figure 3).

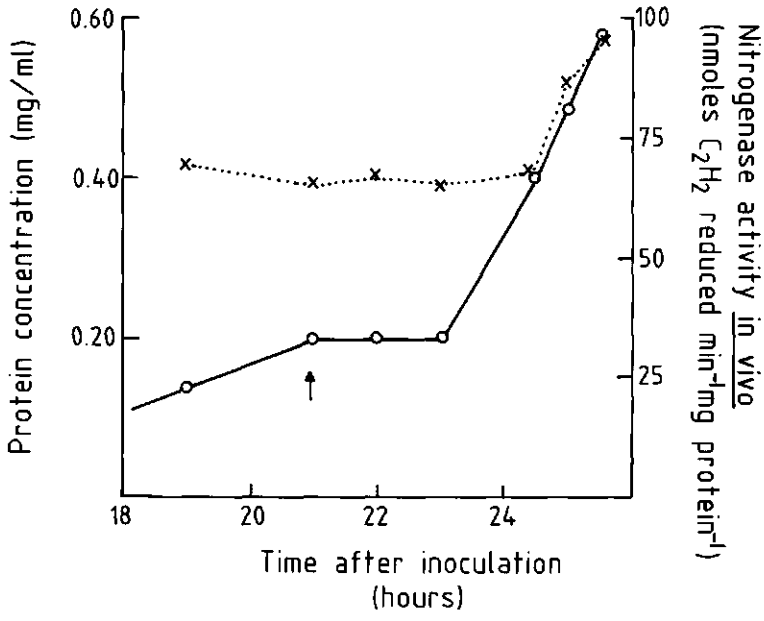

Figure 3. Nitrogenase activity of whole cells before, during and after an oxygen shock. Samples were taken from the fermentor and the maximal nitrogenase activity was determined in a separate system at optimal oxygen input rates.
At the arrow, aeration in the culture was increased, so that the free oxygen concentration increased to 90% of air saturation. o-o, protein concentration of the culture; x-x, nitrogenase activity *in vivo* expressed as nmoles C_2H_2 reduced.min^{-1}mg total cell $protein^{-1}$.

Since Fe-protein is much more oxygen sensitive than MoFe-protein [16], oxygen damage of whole cell nitrogenase activity will affect mostly Fe-protein. When the oxygen input rate of an Azotobacter culture under O_2-limitation is raised to air saturation, nitrogenase activity is inhibited [21]. When subsequently the oxygen input is lowered or when the cells adapt themselves to the high oxygen input rates [22], nitrogenase activity is restored and growth starts again. In the experiment described in figure 3, cells were taken from the fermentor and whole cell nitrogenase activity was measured in a separate system [15] at different oxygen input rates to determine the maximal nitrogen activity. As can be seen, the maximal nitrogenase activity of whole cells is not inhibited by the oxygen treatment. This means that during an oxygen shock Fe-protein is not inactivated due to the high oxygen concentrations. As can be deduced from the recovery data presented in table 1, Fe-protein isolated at the end (type 2 cells) or after an oxygen shock (type 3 cells) is more labile during purification than Fe-protein from logarithmically grown cells (type 1 cells). This indicates that the Fe-protein is altered by oxygen but it still can function *in vivo*.

Discussion

As shown in table 1, it is clear that from cultures with renewed logarithmic growth after an oxygen shock, Fe-preparations can be isolated with an iron/sulphide content above 4. Unfortunately most of these isolations turned out to be labile. Freezing, thawing and anaerobic storage at $20^{o}C$ significantly affects the activity and the iron and sulphide contents of these preparations. One can ask the question if Fe-protein with an iron/sulphide content above 4, is a different protein as regular protein or are the two proteins (polypeptides) similar but the prosthetic groups different or is the amount of apoprotein different. To answer these questions one must realize what happens during an oxygen shock. At the onset of an oxygen shock the nif genes are not transcribed [21], so if there is *de novo* synthesis, it must be at the end of the oxygen shock. From derepression experiments, where *A.vinelandii* cells are transferred from an ammonia containing medium to a medium without a nitrogen source, it takes 30 minutes

Table I.

Iron and sulphide contents of purified Fe-protein isolated form A.vinelandii cells grown under different oxygen conditions. Methods of isolation, Fe and S^{2-} determinations and activity measurements as described in Materials and Methods.

Growth conditions	Specific act. (nmoles C_2H_2 reduced.min^{-1} mg protein^{-1})	Number of iron atoms per molecule Av_2	Number of sulphide atoms per molecule Av_2	Recovery of activity (%)
Logarithmically grown cells (type 1)	1) 2800 ± 200	3.5 ± 0.2	3.9 ± 0.2	70
	2) 2700 ± 200	3.4 ± 0.2	3.8 ± 0.2	
	3) 2600 ± 200	3.2 ± 0.2	3.9 ± 0.2	
Oxygen shocked cells (type 2)	1) 1350 ± 100	3.8 ± 0.2	n.d.	20
Logarithmically grown cells after an oxygen shock (type 3)	1) 2800 ± 200	5.0 ± 0.2	5.0 ± 0.3	50
	2) 1700 ± 140	5.4 ± 0.3	5.1 ± 0.3	
	3) 1600 ± 150	3.4 ± 0.4	5.3 ± 0.3	
	1) *2250 ± 200	n.d.	n.d.	
	2) *1890 ± 170	5.4 ± 0.4	5.1 ± 0.3	
	3) *1875 ± 150	5.7 ± 0.2	5.7 ± 0.1	

1) Directly after the molecular sieve as last purification step.
2) After freezing, storage in liq. N_2 and thawing.
3) Like 2, but with additional concentration on DEAE-cellulose.
Treatments 1, 2 and 3 performed on one preparation.
*Protein preparations from different cultures.

for the synthesis of active nitrogenase (J. Klugkist, personal communication). Since we do not observe a lag period in whole cell nitrogenase activity during or after an oxygen shock and since the increase in biomass after an oxygen shock in the experiments presented here is only 15%, it seems unlikely that under these conditions the cells have completely renewed their set of nitrogenase proteins. In case of de novo synthesis it is possible that the second copy of the nitrogenase genes is transcribed [23-25], but on two-dimensional gels no difference has been found between Fe-protein isolated from oxygen shocked cells and Fe-protein isolated from regularly grown cells (results not shown). This means that at least on two-dimensional gels the Fe-protein peptide isolated from oxygen shocked cells (type 2 and 3) is not different from regular Fe-protein preparations isolated from type 1 cells. The whole cell measurements indicate that Fe-protein in type 2 or 3 cells is physiologically active (fig. 3), but the properties of isolated proteins (table 1) indicate that Fe-protein is altered during an oxygen shock. The simplest explanation is to assume that the Fe-S cluster of Fe-protein is altered by oxygen. When nif gene expression is again possible the protein is repaired by introducing a new Fe-S cluster in all apoprotein molecules present. This results in a maximal Fe-S content of the protein. Since the protein preparation still contains partly damaged protein the total preparation is not as stable as regular Fe-protein, but in vivo this protein might be fully active [26].
We have published earlier that highly active Fe-protein preparations contain up to 8 iron and sulphide atoms per molecule Av_2 [13]. All the different preparations were isolated from one culture of 2500 liters. Six hours before the first cells were harvested, the whole culture stopped growing due to an increase in the stirring speed. During harvesting the air-input was lowered 10% every 500 l. and the remaining cells continued to grow oxygen-limited. Since it took 24 hours before the whole culture was harvested, the cells harvested first were different from those harvested at the end. Only from the early harvested cells, Fe-protein preparations with a high iron and sulphide content were isolated. With our well-defined cultures, the highest number for both iron and sulphide atoms per molecule was 5.7. Unfortunately this protein preparation was labile and the activity was measured only after freezing and thawing; the activity was 1875 nmoles C_2H_2 reduced.min^{-1}.mg Fe-protein^{-1}.

From the isolations of the industrial fermentation we know that it is possible to obtain relatively stable Fe-protein with an Fe/S content around 8 (chapter IV of this thesis). Further studies on this subject will reveal whether the maximum Fe/S value is 8 as stated in chapter IV or is 6 as can be concluded from this chapter. It is important to know the answer because this will lead not only to a complete understanding of the true nature of the Fe/S cluster in Fe-protein, but it might also change the ideas about the mechanism of action of nitrogenase.

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APPENDIX

Remarks about the iron and sulphide analysis upon Fe-protein.

Iron determination

It has been stated before that the analysis of the metal content of nitrogenase proteins has been a frustration for all workers in the field [27]. This thesis is not an exception to this rule; we should like to extend it even to the sulphide analysis as well.

In the literature the molar absorbance of the iron chelator bathophenantroline disulfonate (BPS) is given as 22 $mM^{-1}.cm^{-1}$ [28]. However, we found that this value varies between 18 and 23 $mM^{-1}.cm^{-1}$. Thus a standard calibration curve was always included in our analyses. The wet ashing procedure is hampered by a high blank value and if the strong acids are not added from a previously mixed stock solution (1:1 mixture of HNO_3 and HCl), considerable scattering occurs. The outcome of the iron assay should be the result of at least 5 measuring points in order to be statistically reliable. The Lovenberg degradation extended with an SDS step as described in this chapter is to our experience the most reliable method and for most protein preparations in accordance with the sulphide analyses and wet-ashing method.

The iron content was determined as follows: In a serie of tubes (at least 5) containing different amounts of iron and another set of tubes with the same added iron concentrations and concentration of Fe-protein, iron was determined as described in Materials and Methods. When the results are plotted, two parallel straight lines must be obtained. Quenching effects by the protein can easily be traced; in that case the obtained straight lines are not parallel.

Sulphide determination

The sulphide analysis usually gives reproducible calibration curves. King and Morris did not include a standard with every assay because of the reliability of the method [29]. In general this seems to be true, but similarly as with the iron analyses there can be a significant variation. Since sodium sulphide is hygroscopic, stock solutions should be calibrated iodometrically [30].

Fe-protein might quench the sulphide assay, therefore the same procedure of plotting the results was applied as was used in the iron analysis. S^{2-} can be oxidized to S^0 during the determination. To check this possibility the assay was performed as described by Beinert [17]. For a particular preparation with an iron content of 3.3 Fe/Av_2 this method gave respectively 6.3; 23.5; 22.4 atoms $(S^{2-} + S^0)/Av_2$ (each value is the mean of six points), while the S^{2-} determination as regular gave only 3.6 and 2.8 S^{2-}/Av_2. One must realize that almost all Fe-protein solutions contain dithionite. Apparently this method cannot be used, probably due to degradation products of dithionite from oxidation by Av_2. When the S^{2-} analysis was performed anaerobically, a 10% increase was found (results not shown). This indicates that some oxidation of S^{2-} occurs during the assay. Normally the S^{2-} is liberated by a 30-120 minutes incubation in 110 mM NaOH and 0.74% $Zn(Ac)_2$. To be sure that all S^{2-} is freed, we included a 10 minutes 80^oC incubation in 110 mM NaOH. After cooling, we added $Zn(Ac)_2$ (final concentration 0.74%). After 30 minutes the assay was executed as described [13]. It is possible that after thawing of a highly active protein preparation, from which it is known before freezing that the preparation has high iron and sulphide contents, only the high iron content, can be measured. The sulphide content dropped to 3-4 S^{2-} atoms/Av_2. In these cases the heating procedure increased the S^{2-} content to its original value in agreement with the view that sulphide atoms can be relatively tightly bound (see Table 1). With low active protein preparations with only 3-4 iron and sulphide atoms per molecule, the heating procedure gave the same results as the regular method did. No influence was found upon the calibration curve from the buffer used (Tris-HEPES), dithionite, cysteine or methionine. This

procedure did not effect the outcome in the case of Av_1 or the hydrogenases of Megasphaera elsdenii and Desulfovibrio vulgaris. We do not use this modified analysis since it was not clear to us what exactly the underlying reasons are for the discussed results.

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CHAPTER VI.

The importance of quantitative Mössbauer spectroscopy of MoFe-protein from *Azotobacter vinelandii*

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The Mössbauer spectra of MoFe-protein of *Azotobacter vinelandii*, as isolated under dithionite and taken at temperatures from 125 K to 175 K, are the sums of four resolved quadrupole doublets. Our results indicate that the currently accepted interpretation of these doublets can be questioned. Our data reduction method converts the Mössbauer transmission spectra to source lineshape deconvolved absorption spectra linear in iron. We used these absorption spectra to determine the stoichiometry of the Fe clusters in MoFe-protein and we obtained much better fits if we assumed that there are four iron atoms in the 'Fe^{2+}' doublet, two iron atoms in the 'S' doublet, twelve iron atoms in the 'D' doublet and sixteen iron atoms in the 'M' doublet. Therefore we propose that the MoFe-cofactor contains one molybdenum and eight iron atoms ('M').

We also argue that none of the previous Mössbauer spectroscopic studies have been performed on the highest-activity preparation now obtainable, nor has there been any study to prove that the Mössbauer spectra are independent of activity. We consider that the Mössbauer spectroscopic studies of the MoFe-protein of nitrogenase are a re-opened and unsolved problem.

The determination of the transition metal distribution within the MoFe-protein component of nitrogenase has largely been the result of Mössbauer spectroscopic experiments on samples from *Klebsiella pneumoniae* [1] and *Azotobacter vinelandii* [2]. The purpose of this paper is to demonstrate a weakness in these interpretations, to offer an alternative explanation, and to outline the method by which we feel this problem should be solved. By the latter, we mean that not only are the Mössbauer spectra for the MoFe-protein susceptible to large distortions during data reductions, but also that the samples that were used in the previous studies are not of the highest activity achieved in today's experiments, and are, therefore, not necessarily representative of the true metal content of the MoFe-protein *in vivo*.

The nitrogenase complex consists of two protein units: the MoFe-protein and the Fe-protein. The MoFe-protein (M_r = 220000) has two subunits of 50 kDa and two subunits of 60 kDa. It contains the active site for the reduction of dinitrogen; fully active MoFe-protein contains 2 Mo atoms and 24–32 Fe and S atoms per complex [3, 4]. The Fe-protein is a dimer (M_r = 63000 total); it reduces the MoFe-protein in the presence of MgATP, and it is reported to have four Fe and four S atoms per dimer [3] but recent studies indicate that higher contents are possible [5]. MoFe-protein has been studied by EPR and Mössbauer spectroscopies. Smith et al. [1, 6] studied Kp_1 and Kp_2 and Münck et al. did an extensive study on Av_1 [2, 7, 8] and Cp_1 [9]. From those studies, it was proposed that the MoFe-protein has two FeMo-cofactors [1 Mo-6 Fe-6 S], four P clusters [4 Fe-4 S] and two iron atoms in an unknown arrangement, possibly in a [2 Fe-2 S] cluster [6]. The FeMo-cofactor has been isolated from the MoFe-protein [10] and studied by Mössbauer spectroscopy [11]. A recent determination of the stoichiometry of the MoFe-cofactor has been performed by a radiochemical method. The observed stoichiometry is 1 Mo:8.2 Fe:8.7 S [12]. In the presence of dithionite, preparations of Av_1 show an EPR signal that has been attributed to an $S = 3/2$ spin state of the FeMo-cofactor [2]. ENDOR studies of the dithionite-reduced MoFe-protein have assigned the Mo atom to the $S = 3/2$ signal [13]. Furthermore, recent ENDOR results have tentatively identified six unique iron sites, with the projections of their magnetic hyperfine tensors along the principal axes of the $S = 3/2$ g-tensor [14]. The substitution of these values into a Mössbauer spectroscopic synthesis program (Hoffman, B. M., personal communication) appears to result in good fits and to confirm the previous assignments by the Mössbauer spectroscopists [2, 7–9].

For the purposes of this article, it is necessary to discuss only the high-temperature (125–175 K) Mössbauer spectra of the MoFe-protein (as isolated) from *Azotobacter vinelandii* (Av_1). These Mössbauer spectra are comprised of four quadrupole pairs. We will use the previously defined [2, 15] nomenclature of these pairs: 'M' for the iron atoms in the FeMo-cofactor, 'D' for the larger iron component of the P clusters; 'Fe^{2+}' for the smaller iron component of the P clusters; and 'S' for the remaining doublet in the spectra. The

Abbreviations. The MoFe and Fe-proteins of the nitrogenases of *Azotobacter vinelandii*, *Clostridium pasteurianum* and *Klebsiella pneumoniae* are referred to as Av_1 and Av_2, Cp_1 and Cp_2 and Kp_1 and Kp_2 respectively. Av Fe/S II stands for Fe/S protein II from *A. vinelandii*; EPR, electron paramagnetic resonance; ENDOR, electron nuclear double resonance.

Enzyme. Nitrogenase (EC 1.18.2.1).

question of central importance is: How many iron atoms are represented by each doublet?

MATERIALS AND METHODS

Growth conditions and enzyme preparation

Azotobacter vinelandii ATCC 478 was grown in the standard Burk's nitrogen-free basic salt medium in a batch culture as described earlier [5] except that $FeSO_4 \cdot 7\ H_2O$ was replaced by 1 mg/ml ^{57}Fe.

Isolation of the nitrogenase proteins and standard nitrogenase activity assays were run as described earlier [5]. Av_1 and Av_2 purified by the described procedure had specific activities of 2000 – 2400 nmol C_2H_4 formed · min^{-1} · (mg MoFe-protein)$^{-1}$ and 1800 – 2000 nmol C_2H_4 formed · min^{-1} · (mg Fe-protein)$^{-1}$. The activity of MoFe-protein was not enhanced when its activity was measured with Fe-protein with more than four Fe atoms and four sulfide atoms/molecule [5]. The MoFe-protein used in this study had a specific activity of 2250 nmol C_2H_4 formed · min^{-1} · mg Av_1^{-1}. The Mo, Fe and S^{2-} content per mg Av_1 was 7.3 ± 0.4 nmol Mo, 109 ± 9 nmol Fe and 100 ± 9 nmol S^{2-}. The molar ratio of Mo:Fe:S^{2-} per molecule Av_1 is 1.6:24:22.

Analytical methods

The Mössbauer spectra were taken on a previously described spectrometer [16] with a Cryogenics Associates dewar and a highly modified Ranger Instruments drive. We have recently added a type II servo mechanism to the feedback loop of the drive [17]. Further papers characterizing the servo loop performance and design are presently in preparation. The drive routinely demonstrates an accuracy and precision of less than ± 0.001 mm/s over the entire span of a 24-h experiment. We know of no other spectrometer that can match this short or long-term stability performance specification. Furthermore, the long-term stability is also demonstrated by the 125 K spectra in this article, whose data were collected over a six-week period due to the low count rate from our 2-year-old 35-mCi ^{57}Co-in-rhodium source.

Molybdenum [18], iron [19] and sulphide [20] were determined using published procedures. Protein concentration was estimated with the micro-biuret method of Goa [21] with bovine serum albumin as the standard.

Materials

ATP, creatine kinase (EC 2.7.3.2), creatine phosphate, and bovine serum albumin were obtained from Sigma Chemical Co; all other chemicals were of the highest commercial grade. Argon was purified by passage over hot (150 °C) BASF catalyst.

RESULTS

Fig. 1A shows the raw data from a 125 K Mössbauer spectrum of Av_1 corrected only for pulse pile-up and solid angle effects [16]. It is, therefore, suitable for comparison with data from other MoFe-protein preparations. If the background counts due to non-resonant 14-keV gamma rays and Compton events in the gas counter are subtracted, Fig. 1B is obtained with an increase in the percentage effect from the background correction. If then the spectrum is deconvolved by the source lineshape [16], Fig. 1C results again with a significant increase in the percentage effect. The mathematical process that performs this deconvolution requires a truncation in Fourier space; therefore, there are ripples in the original space version of this spectrum (Fig. 1C). Others [8, 9] have also used Fourier deconvolution on their spectra, but the method of Ure and Flinn [22] used by these authors is imprecise and results in an error that makes it useless in the following step.

In this step, the logarithm of the deconvolved data was taken, giving the result shown in Fig. 1D. Note that the maxima are well into the region where $e^{-x} \neq 1-x$. Thus, data treatments that ignore this step have large line distortions. The Fourier deconvolution method of Ure and Flinn [22] also should give a spectrum corresponding to Fig. 1D. However, in that method, the Fourier deconvolution step is stabilized in Fourier space with multiplication by a Gaussian lineshape. This procedure is an explicit convolution, whose side effect is that it prevents one from accurately taking the logarithm of the data in the following step. The process of taking the logarithm of a function to be Fourier-transformed is mathematically complicated [23]. In order to stabilize the Fourier space one needs to set to zero those values where the deconvolved noise in the Fourier space is much larger than the deconvolved signal by multiplying them by a 'filter' function. Any convex function can be made to act as a filter function including the Gaussian function. On the other hand, one needs to leave unchanged those values in Fourier space where the signal/noise ratio is higher than unity, so that taking the logarithm of the backtransformed spectrum has the maximum chance to generate a valid representation of the signal. Truncation multiplies by one those elements in Fourier space where signal/noise is greater than one and multiplies by zero those elements where it is less than one. Truncation is, therefore, the ideal filter. The method of Ure and Flinn requires the use of a filter function that greatly modifies some of the significant elements in Fourier space and at least weakly modifies all the elements of the Fourier space so that their method can be much less accurate than our method [16, 17, 24]. Furthermore, our minimization (curve-fitting) procedures take place in the Fourier space so that they are almost unaffected by the truncation.

In Fig. 2A, the backtransform is shown of one of these fits superimposed on the data with the difference spectrum below (Fig. 2B). If the 'ripples' in the data and simulation are bothersome, we suggest that one concentrate on the difference spectrum where these ripples are absent. (The periodic nature of the difference spectrum also results from truncation in Fourier space, but these ripples are due to 'noise' if the fit is perfect.) In the fit, as shown in Fig.2A, we allow the isomer shifts and quadrupole splittings of four independent quadrupole pairs to vary until a best fit is obtained, while fixing the relative intensities of the four pairs to the following ratio: 4:2:12:12. This is the currently accepted model for the MoFe-protein: 4 iron atoms in center 'Fe^{2+}'; 2 iron atoms in 'S'; 12 iron atoms in 'M'; and 12 iron atoms in 'D'. The fit is not bad, but it is much worse than the fit shown in Fig. 2C. The difference spectrum is shown in Fig. 2D. In this fit, the iron ratios are 4:2:16:12.

In Fig. 3 the data and fits with ratios 4:2:16:12 for 150 K and 175 K are shown. All the lines in these simulations have the same linewidth (FWHM) of 0.16 mm/s. The lineshape should be the convolution of a Heissenberg Lorentzian lineshape and the frequency function for the Mössbauer

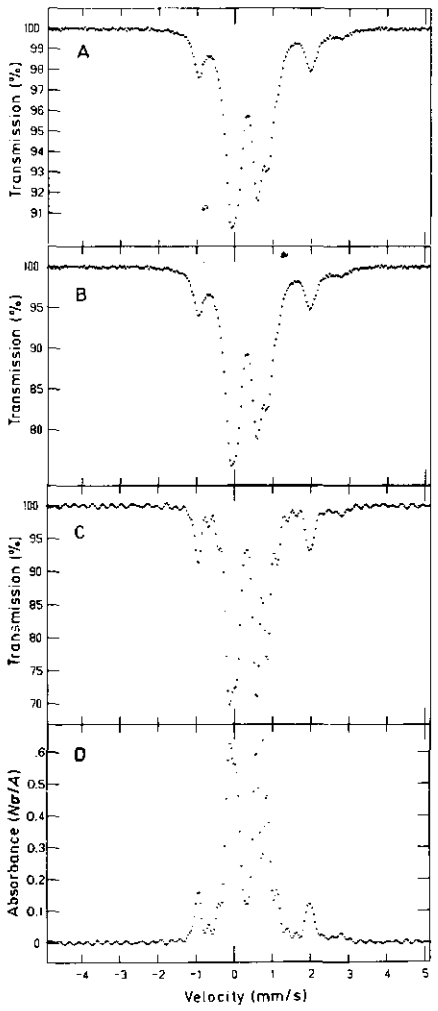

Fig. 1. *Mössbauer data for Av_1 as isolated, at 125 K and zero applied magnetic field.* The isomer shifts are relative to iron metal at 298 K and corrected to a 298 K standard absorber state using the Debye model with Θ_D = 180 K. Av_1 (21.9 mg/ml) was isolated as described in Materials and Methods. (A) Raw data, corrected for pulse pile-up and solid angle effects. (B) Data corrected for background due to nonrecoil-free gamma rays in the beam and Compton events in the counter. (C) Background-corrected data deconvolved by the source lineshape. (D) Background-corrected and deconvolved data, plotted as logarithmic values

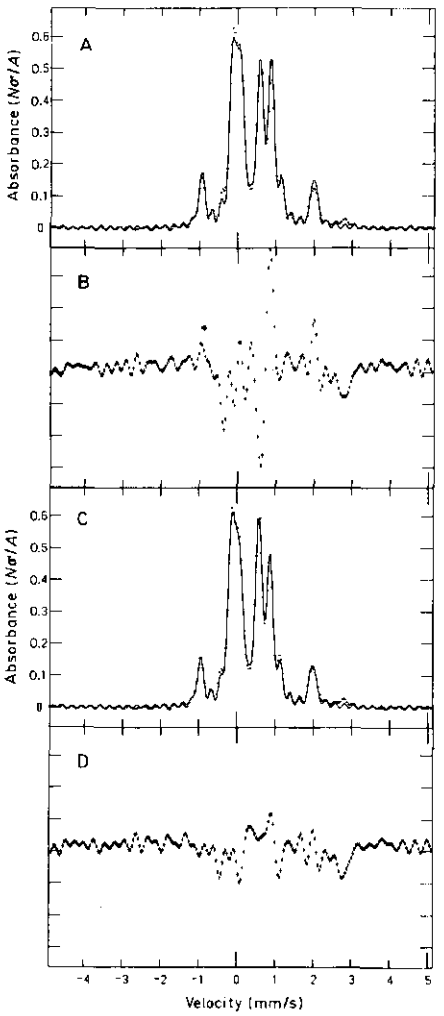

Fig. 2. *Mössbauer data and fits for Av_1, as isolated at H = 0 and 125 K.* Av_1 (21.9 mg/ml) was isolated as described in Materials and Methods. (A) Data (+) and fit (——) where iron ratios Fe^{2+}:S:M:D are 4:2:12:12. (B) Fit minus data for A, magnified 5 × vertically. (C) Data (+) and fit (——) where ratios are 4:2:16:12. (D) Fit minus data for C, magnified 5 × vertically

parameters distribution. The use of a Lorentzian lineshape, although not formally correct, does not result in an error larger than the noise in this experiment when the width is this small (0.16 mm/s). The 4:2:16:12 simulations have the same integrated total intensity to an error of less than 1%. We expect these numbers to be more accurate than any intensity numbers that we can derive directly from the data because they are less sensitive to baseline error. Both the absorber Debye-Waller factor and the second-order relativistic Doppler shift corrections have been included for the absorber under the Debye model with a Debye temperature of 180 K.

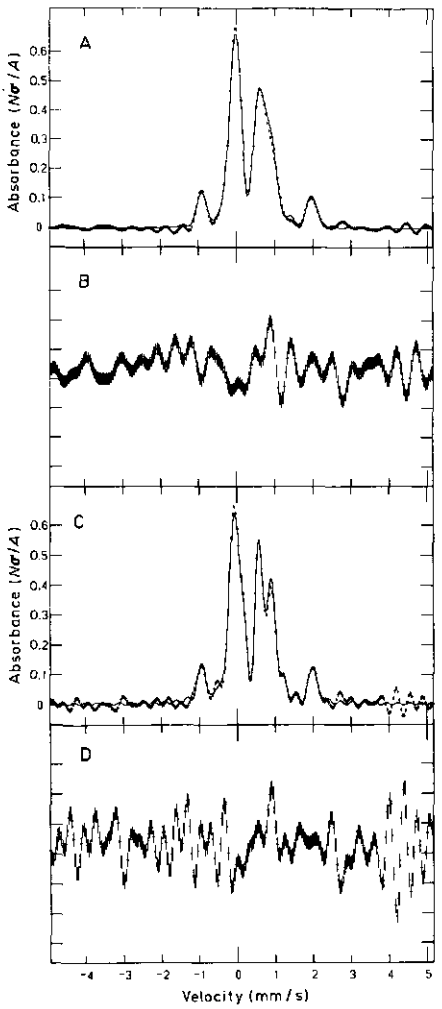

Fig. 3. *Mössbauer data (+) and fits (——) for Av_1 as isolated, at* $H = 0$. Av_1 (21.9 mg/ml) was isolated as described in Materials and Methods. The iron ratios Fe^{2+}:S:M:D are 4:2:16:12. (A) 175 K; (B) fit minus data for A, magnified 5× vertically; (C) 150 K; (D) fit minus data for C, magnified 5× vertically

Therefore, the agreement in total intensity is simultaneously a demonstration of the precision of the data reduction procedure and a validation of the Debye model over this temperature range. We chose this particular temperature range because our experience has been that in this range proteins follow a Debye model well, that they are too cold to demonstrate the onset of the 'liquid' state (> 200 K), and that they are too warm to show low-temperature complications

Table 1. *Mössbauer data on MoFe-protein and Fe/S protein II from* Azotobacter vinelandii

The isomer shifts in this table contain a correction for the second-order relativistic Doppler shift [16]. To remove this correction, subtract 0.087 mm/s (175 K), 0.104 mm/s (150 K) or 0.121 mm/s (125 K)

Spectral component	Temperature	Isomer shift	Quadrupole splitting
	K	mm/s	
Av_1 'Fe^{2+}' doublet	125	0.516	2.941
Av_1 'Fe^{2+}' doublet	150	0.526	2.935
Av_1 'Fe^{2+}' doublet	175	0.532	2.900
Av_1 'S' doublet	125	0.444	1.377
Av_1 'M' doublet	125	0.234	0.690
Av_1 'M' doublet	150	0.239	0.697
Av_1 'M' doublet	175	0.249	0.674
Av_1 'D' doublet	125	0.472	0.792
Av_1 'D' doublet	150	0.485	0.808
Av_1 'D' doublet	175	0.475	0.786
Reduced Av Fe/S II	125	0.119	0.96
		0.504	2.863

from Debye-Waller factor anomalies and magnetic splittings. In all three fits with this model (Fig. 2C, 3A, 3C) the difference spectra resemble pure noise with the exception of the position of component X, most easily seen in Fig. 3A. We feel that this line is the high-velocity line of a quadrupole doublet ($\delta = 1.4$ mm/s, $\Delta E_q = 2.8$ mm/s) seen before in the Mössbauer spectra of spinach and *Chromatium* ferredoxin [25] and some preparations of Kp_2 [6]. This spectrum is similar to the one from $FeSO_4 \cdot 7\,H_2O$ [26] and therefore, probably represents a degradation product between the $S_2O_4^{2-}$ reaction products and a low-pH form of the protein Fe/S centers. The maximum height represented by component X is one-half of an iron atom. Therefore, we do not feel that the presence of X in these spectra significantly affects the difference spectra.

DISCUSSION

We have shown in this paper that the Fe distribution in MoFe-protein can be better explained by a 4:2:16:12 stoichiometry than by a ratio of 4:2:12:12 as suggested in the literature. The different stoichiometry is mainly a consequence of our advanced data reduction method since the raw Mössbauer data are not significantly different from those published by others [1, 2, 6–9, 28]. We emphasize that our data reduction procedures are most critical when a Mössbauer spectrum has widely varying (weak and strong) line intensities as in the case of reduced MoFe-protein at high temperature. There are several reasons why we think that the problem is as yet unsolved even though the quality of our fits is extremely high. The specific activity of our protein sample was 2250 nmol C_2H_4 formed $\cdot$ min^{-1} $\cdot$ (mg $Av_1)^{-1}$ as compared to 1200–1650 nmol C_2H_4 formed $\cdot$ min^{-1} $\cdot$ mg^{-1} for samples of Cp_1 and Av_1 [2, 9] and 1000–1400 nmol C_2H_4 formed $\cdot$ min^{-1} $\cdot$ (mg $Kp_1)^{-1}$ [1, 6]. The specific activity of the sample studied here is good; however, samples with a specific activities up to 3000 nmol $\cdot$ min^{-1} $\cdot$ mg^{-1} have been obtained [27]. All Mössbauer data of MoFe-proteins are extrapolated to fully active protein with a Mo content of 2 Mo atoms and

30–36 Fe atoms in a molecule of 220 kDa. These extrapolations assume an 'all-or-non' mechanism for metal-loss from the MoFe-protein upon inactivation. If MoFe-protein denatures in an 'all-or-none' fashion, then the data in this article represent the fully active *in vivo* protein; in that case, there are eight iron atoms in the FeMo-cofactor of nitrogenase, not six.

Our interpretation of these data raises the question: can there by 7 or 9 irons per molybdenum in the cofactor? The answer is yes. The uncertainty in the total iron content of current Av_1 preparations (30–36) means that all stoichiometries for the iron clusters are uncertain to one iron atom. However, one of the major points of this paper is to emphasize that some Mössbauer research on nitrogenase is favouring eight irons per FeMo-cofactor rather than the currently accepted number, six. The chemical analysis for the isolated FeMo-cofactor usually gives an iron to molybdenum ratio around 8 [10, 12], except when the isolation procedure includes an iron chelation step [29]. Our tentative conclusion that the FeMo-cofactor in 'the' MoFe-protein always contains 8 Fe per Mo implies that the recent analysis of 'Fe' ENDOR from Av_1 is incomplete [14].

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Chapter VII. DISCUSSION

A. ON THE NATURE AND ON THE PROPERTIES OF THE [Fe-S] CLUSTER(S) OF Fe-PROTEIN OF NITROGENASE

State of knowledge in 1980

Immediately after the first successful isolation of the nitrogenase proteins [1], studies were undertaken to understand the mechanism of action of nitrogenase. Since 1973 there is a general consencus on the flow of electrons through nitrogenase: namely an electron donor (flavodoxin or ferredoxin) reduces the Fe-protein; reduced Fe-protein transfers its electron to the MoFe-protein and substrate reduction takes place on the MoFe-protein. This concept was mainly deduced from experiments where the redox state of Fe-protein and that of MoFe-protein were monitored by EPR spectroscoy [2-4]. Already during the formulation of this concept, studies were undertaken to understand the redox properties of both nitrogenase proteins. From these studies a working hypothesis was formulated for Fe-protein by Orme-Johnson *et al*. [5] (see the introduction of chapter 3) and after that time (1976) there seems to be a general agreement in the literature about the structure and redox properties of Fe-protein. In this hypothesis Fe-protein contains a ferredoxin type of [4Fe-4S] cluster which acts as a one-electron donor/acceptor. This cluster is sandwiched between the two identical polypeptides of the dimeric Fe-protein.

With respect to the literature available in 1980, when this thesis work was started, two observations were not explained satisfactory.

1° The low integration of the EPR signal of Fe-protein (0.2-0.3 unpaired electrons per Fe-protein molecule) was not elucidated satisfactory because experimental evidence for the presence of the postulated second paramagnet was lacking.

2° Why were the original data about the Fe-protein being a 2 electron donor not correct [6,7]?

In the rest of this chapter, both questions will be discussed with respect to the results obtained during this study about the nature of the [Fe-S] clusters present in Fe-protein of *A.vinelandii*.

The physical nature of the iron/sulphur cluster that causes the EPR signal of Fe-protein

It is generally assumed that the EPR signal of Fe-protein is associated with a ferredoxin type cluster e.g. a [4Fe-4S] cluster. The evidence is summarized in the introduction of chapter III. The first question which emerges when one critically thinks about the physics/inorganic chemistry behind the nature of the EPR signal of the Fe-protein, is whether this EPR signal is a signal of a redox group in the protein that can be reversibly reduced and oxidized. I think that there is hard evidence that this is the case. Fe-protein shows under reducing conditions (in the presence of dithionite) an EPR signal, while dye-oxidized or physiologically oxidized Fe-protein is EPR silent. When Fe-protein and MoFe-protein were mixed in the presence of dithionite, the EPR spectrum of the mixture was the sum of the two spectra. When MgATP was added, the intensity of the MoFe-protein spectrum decreased strongly and after dithionite had been consumed, the original spectrum of MoFe protein was restored but the Fe-protein spectrum had disappeared completely [2-4]. From these experiments it was concluded that electrons are transferred from reductant via Fe-protein to MoFe-protein and that the EPR signal represents the reduced state of the [Fe-S] cluster present in Fe-protein. One serious problem arises when the signal of Fe-protein is integrated. The intensity of the signal is mostly about 0.3 electron.molecule^{-1}, which is far below the expected value of 1.0 [8]. This low integration value has been explained by assuming a second rapidly relaxing paramagnetic EPR-invisible redox centre which is in magnetic interaction with the EPR-visible [4Fe-4S] cluster [9]. We have obtained evidence that argues against the assumption by Lowe and makes the existence of a second paramagnetic centre very unlikely. PIXES experiments have been performed on our preparations to see whether there are other metal ions present which could account for an EPR-invisible centre, but apart from iron, no other metal ions were found to be present in Fe-protein (Chapter IV).

Hagen et al. [10] studied the EPR signal of Fe-protein in detail. It was found that: 1^{o} The integral of the EPR signal is inversily proportional with the absolute temperature (Curie's law). 2^{o} Intensity behaviour upon

continuous power saturation is usual for [4Fe-4S] cluster, so there is no fast relaxing species present. 3° There is no half-field signal detectable; a signal which is often present when there is a magnetic interaction. 4° The signal shape (linewidth) is independent of the microwave frequency applied from 9-15 GHz. 5° Simulation based upon the theory of g-strain, assuming a magnetically isolated s=½ system, results in an acceptable fit which is much better than the computer simulation fits published earlier [9].

From this evidence there is only one conclusion possible, namely: the EPR signal arises from a regular isolated s=½ spin system very similar to a ferredoxin type of [4Fe-4S] cubane. This means that an EPR integral of 0.3 electrons per molecule Fe-protein indicates that 30% of the enzyme molecules has a [4Fe-4S] cluster and the other iron atoms are located in a cluster (or clusters) which is (are) not directly visible in EPR. This (these) unknown cluster(s), when extracted from the protein, has (have) the same spectroscopic properties as the clusters found in ferredoxin [11,12]. If the [4Fe-4S] cluster is a degradation product of such unknown cluster(s), then a reverse relation between the value of the EPR integral and the specific activity is expected. However, neither in the literature [5], nor in our own work can we find such a correlation.

Methods used to detect the EPR invisible iron atoms in Fe-protein

The obvious method to monitor all iron atoms in a protein is Mössbauer spectroscopy. This technique sees all the iron atoms regardless of the oxidation state, while magnetic and non-magnetic species can be distinguished, coupling constants and their signs can be determined so that antiferromagnetism can be detected. Furthermore the molar X-ray resonant absorbance of all iron atoms at low temperatures seems to be about the same.

Our own results (work done in cooperation with Dr. W. Dunham and Dr. W. Hagen) indicate that in both the reduced and the oxidized Fe-protein, irrespective of the iron/sulphide content, at temperatures from 125-175°K) all iron atoms are equivalent. In case of reduced protein the iron atoms are all in a paramagnetic cluster. Also no influence of MgATP or MgADP was

found upon the Mössbauer spectrum of Av_2. This is in sharp contrast with EPR experiments. These adenine nucleotides have the following effects. When the redox state of the EPR active cluster is monitored by the intensity of its signal then causes the binding of MgATP or MgADP an apparent shift in midpoint potential; a change of the n-value (in case of MgADP), and in the case of binding MgATP the g-value anisotropy of the EPR spectrum decreases (Chapter II). In contrast to this, when the redox state of the protein is monitored by its absorption at 425 nm, no influence of adenine nucleotides is seen (Chapter III).

Since Mössbauer and the absorption at 425 nm are monitoring all the iron atoms in the Fe-protein irrespectively of the presence of an unpaired electron, while EPR follows only the [Fe-S] cluster causing the $s=\frac{1}{2}$ spin system, it is clear that only a minority of the Fe-atoms present in Fe-protein is monitored by EPR spectroscopy. From our work it is also clear that the midpoint potentials of the EPR visible and the EPR invisible clusters are different.

Fe/S content of Fe-protein

During the study of the redox behaviour of Fe-protein it was noted that the iron content of the enzyme preparations was sometimes more than four atoms per molecule. Further research focussed upon the iron- and acid labile sulphur contents, revealed that Fe-protein can have indeed more than four iron and sulphide atoms per molecule (Chapter IV). In Chapter V it is demonstrated that the growth conditions can determine the iron- and sulphide contents, but we found no significant differences between the shape and intensity of EPR spectra of Fe-protein preparations with 4 and 6 iron atoms per molecule (results not shown). From the evidence available now (Chapter V) the Fe-protein polypeptide can accommodate a variable amount of Fe/S atoms.

Further evidence that the Fe-protein does not contain a simple cubane comes from electron counting experiments (Chapter III). From these experiments it can be derived that it is very likely that two electrons can be taken up by dye-oxidized or by physiologically oxidized Fe-protein. These

results are in accordance with the values reported earlier [4,5], but in contrast with others [13]. At the moment we have no explanation for the differences found. The observation that two electrons are taken up by the Fe-protein is not compatible with the postulate that it contains only one regular [4Fe-4S] cluster.

Redox dependent activity reactions also give n-values above one. In these experiments (Chapter II), care has been taken that there was a redox equilibrium between the electron donors used and the Fe-protein. In this case the activity of MoFe-protein was used to monitor the redox state of Fe-protein. Dependent upon the electron donor used we found n-values ranging from 1.5 for methyl viologen to 2.5 for flavodoxin from M.elsdenii.

It is clear that there are several experiments that cannot be explained by assuming that Fe-protein is a dimer with a single ferredoxin type of [4Fe-4S] cluster sandwiched between the two subunits. We think that Fe-protein preparations are not homogeneous with respect to [Fe-S] cluster composition. Some molecules have a regular [4Fe-4S] cluster, other molecules have another not yet identified [Fe-S] cluster. This cluster might contain more than four iron and sulphide atoms and there is the possibility that there is a significant amount of apoprotein.

The final conclusion of my work on the composition of Fe-protein is that the EPR active [4Fe-4S] cluster is not the main [Fe-S] cluster in Fe-protein and that the true nature of the main cluster in Fe-protein is still unknown. The answer to this question might come from very recent work of Hagen et al. [16]. They found that Av_2 contains a s=3/2 spin system with an EPR signal around g=5. This signal was observed earlier but it was ignored since it was thought to be an Av_1 impurity (Hagen, personal communication).

The relation between the presence of [Fe-S] clusters in Fe-protein and its activity

During my study I found that there is no relation between the intensity of the EPR signal and the specific activity of Fe-protein. Since we have indicated that this [4Fe-4S] cluster represents only the minority

the iron atoms in Fe-protein molecules, it might be possible that there is a relation between the presence of the other [Fe-S] cluster(s) and the specific activity. To clarify this, the first logical thing to do is find a way to detect the other [Fe-S] clusters.

There is circumstantial evidence which might give a hint how to identify the unknown cluster(s). In EPR at low temperatures (6-10^{o}K) another signal was detected, partly hidden under the [4Fe-4S] cluster signal. From differential saturation of the two signals a spectrum was obtained which was not a fingerprint of a common [Fe-S] cluster. Simulation gave g-values similar to those of the recently identified [6Fe-6S] cluster [16]. This synthetic cluster is metastable and easily decomposes into the [4Fe-4S] cluster type which can be assembled spontaneously and is relatively stable. The metastability of this cluster can also be the reason that a [2Fe-2S] cluster EPR signal is found in Fe-protein [15]. Surprisingly there is hardly any difference in the Mössbauer spectra of some of the synthetic [6Fe-6S] and [4Fe-4S] clusters [16].

It would be logical to suppose that the iron and sulphide content are correlated with the specific activity. Because Fe-protein is an electron transferring protein and electrons must be stored at least temporary at the [Fe-S] cluster. In chapter IV we indeed suggest a relation between the specific activity and the iron/sulphide content. However, as can be seen from fig. 1 (chapter IV) there is a considerable scatter in the data points. Probably not enough active preparations with less than 4Fe/S atoms per Av_2 have been isolated during this study. In chapter V we have shown that also highly active preparations can be obtained with only four iron and sulphide atoms per molecule. In dilute activity measurements the rate of dissociation of oxidized Fe-protein from MoFe-protein is the rate limiting step in nitrogenase turnover. So it might be possible that a less functioning [Fe-S] cluster has no immediate effect on the activity. It is also possible that the ATPase activity of Fe protein is determining the specific activity. There are indications that beside the [Fe-S] cluster the ATP binding site is very sensitive to inactivation. In general, oxygen oxidized but not inactivated samples have higher ATP/2e ratio's [17] and less active Fe-protein has altered ATP binding properties [20]. That the absence of activity cannot always be ascribed to a damaged iron-sulphur cluster is shown

by Mössbauer experiments; a Fe-protein preparation with a specific activity of 900 still contained an intact iron/sulphur cluster, (18; unpublished results). Yates found that the inhibitory effect of MgADP upon the reduction of Fe-protein cannot be found anymore when the Fe-protein is inactivated by O_2 [19]. Thus the adenine nucleotide binding sites seem to be affected. It is clear that from the confusing results summarized above one can only conclude that it is not clear which factors determine the specific activity of Fe-protein.

B. MÖSSBAUER STUDIES OF MoFe-PROTEIN OF NITROGENASE.

In Chapter VI recent results of Mössbauer studies upon the MoFe-protein are presented. The stoichiometry of the [Fe-S] clusters in this protein was considered to be known without any doubts [21]. However it is made clear in Chapter VI that one must seriously question this matter. The reasons are:

1. The Fourier deconvolution of the spectra according to the method of Ure and Flinn [22] as was used up to now to analyse the Mössbauer data [23] makes the data unsuitable for further processing.
2. For quantification the transmission data are often transformed to absorption units. This is not always done in the region where the transmission has a linear relationship with the absorption. In our studies we avoided this mistake.
3. All MoFe-protein preparations used in the past were not highly active and did not have the maximal metal content. In the past corrections were made by assuming two molybdenum atoms per active MoFe-protein molecule.

By doing this an iron content of 32-34 iron atoms per molecule MoFe-protein was calculated. Since one is not certain that a loss of metal atoms is an all or nothing process, these extrapolations may not be allowed. For comparison with the literature, we have performed similar extrapolations, but as discussed in chapter VI even then due to our data reduction methods the general accepted stoichiometry of the FeMo-cofactor of nitrogenase must be questioned. To avoid the problems about extrapolation, it is necessary to

obtain Mössbauer spectra of fully active MoFe protein with two Mo atoms per molecule. By growing cells optimal for nitrogen fixation, we succeeded in the isolation of a MoFe-protein preparation with 2 molybdenum atoms, 30 iron atoms and 30 sulphide atoms per molecule. This preparation has a specific activity of 2900 nmoles C_2H_2 reduced.min^{-1}.mg MoFe-protein^{-1}. With this protein preparation no extrapolation is necessary to account for inactive protein. A detailed Mössbauer study of this type of protein will lead in the future to the true stoichiometry of the different [Fe-S] centers present in MoFe-protein.

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SUMMARY

In Chapter VII (Discussion) the current thoughts as they were accepted in 1980 about the electron transport within the nitrogenase complex are summarized. The starting points and ideas about the subject of this thesis are also given. In this way, Chapter VII can be considered both as a summary and as a discussion for the scientific interested reader. For those, who are less familiar with this kind of research, the following summary gives the results of this study which emerge in the different chapters.

In the biological N-cycle, the fixation of atmospheric dinitrogen is a key reaction. The enzyme nitrogenase catalyzes the reduction of dinitrogen to ammonia. The structure and function of nitrogenase isolated from different organisms is very similar, so most conclusions drawn from experiments with one particular nitrogenase can be extended to other nitrogenases as well. In this thesis experiments are described with nitrogenase from Azotobacter vinelandii.

The enzyme is in vitro easily separated into two components, both essential for activity. The smallest component is called the Fe-protein and its function seems to be restricted to reduce specifically the bigger component, the MoFe-protein. This electron transfer only occurs when MgATP is present and MgATP is hydroylzed to MgADP and P_i during electron transfer. The actual reaction, the reduction of dinitrogen to ammonia, takes place on the MoFe-protein.

Both proteins have iron/sulphur clusters, structures consisting of alternating iron and sulphide atoms, where the necessary electrons can be stored. The Fe-protein is thought to have only one iron/sulphur cluster of the [4Fe-4S] type, which is also found in ferredoxins. This kind of cluster can take up or donate only one electron.

Although the experiments, which give rise to this hypothesis, seem very convincing, there are also observations described in literature which are not compatible with the view of one [4Fe-4S] cluster per molecule Fe-protein and a one electron donor/acceptor behaviour in redox reactions.

This discrepancy is also present in this thesis. In chapter II for

instance, redox reactions are described where the redox state of the Fe-protein is followed by means of EPR spectroscopy and by the activity of the intact enzyme complex. From the results obtained it can be concluded that possibly two electrons are involved in the redox reaction of Fe-protein with the MoFe protein. The experiments described in chapter III confirm this view. In these experiments the number of electrons is determined which can be accepted by oxidized Fe-protein. In stopped-flow experiments dye-oxidized Fe-protein could accept two electrons from reduced methyl viologen. Since it is possible that the Fe-protein is over-oxidized by dyes, also the physiologically oxidized Fe-protein has been used. Also in this case the results obtained are more compatible with the view that Fe-protein behaves as a two electron donor/acceptor.

Because of the fact that a [4Fe-4S] cluster can only react as a one electron redox centre, we examined our Fe-protein preparations by means of Proton Induced X-ray Emission Spectroscopy (PIXES) on the presence of other metal ions which could function as an electron acceptor. No other metals than Fe could be detected. But we found a much higher iron content than could be expected by assuming one [4Fe-4S] cluster. This gave rise to a thorough analysis of the iron/sulphide content of Fe-protein (chapter IV). The maximum number of iron and sulphide atoms found per molecule was eight. This combined with the results of Mössbauer experiments which indicate that all iron atoms are in an equal configuration, gave rise to the view that probably two [4Fe-4S] clusters are present in active Fe-protein.

In chapter V, it was shown that the growth conditions determine the iron/sulphide content of isolated Fe-protein. During this investigation the maximum number of iron and sulphide atoms per molecule Fe-protein was six and not eight. The discrepancy between the values reported in chapters IV and V are discussed in chapter V.

Mössbauer spectroscopy experiments were not restricted to the Fe-protein, but also the MoFe-protein has been subject of examination. The importance of our results are not only restricted to the fact that the specific activity of the protein sample used is much higher than that of others, but also the processing of the primary data has been altered. This resulted in a different ratio of Fe to Mo of the FeMo-cofactor of MoFe-protein. Due to this fact the composition of the other iron/sulphur clusters present in MoFe-protein is now questionable.

It is clear that the work described in this thesis has not solved the problem of the composition and structure of the metal-sulphide clusters present in the nitrogenase proteins. Beside the described experiments which may lead to the discovery of the true nature of these clusters, an important aspect is that the general accepted dogma that the structure of the metal-sulphide clusters of nitrogenase has been solved is questioned and reopened the reserach towards a better understanding of the structures and function of these clusters.

SAMENVATTING

In hoofdstuk VII (Discussion) worden de heersende gedachten in 1980 over het electronentransport binnen het nitrogenase enzymcomplex samengevat en wat de uitgangspunten en ideeën waren die aan dit proefschrift ten grondslag lagen. Hoofdstuk VII is dan ook op te vatten zowel als samenvatting en discussie voor de wetenschappelijk geïnteresseerde lezer. Voor lezers, minder thuis in deze tak van wetenschap volgt hieronder een samenvatting van de resultaten van dit promotie-onderzoek zoals die in de verschillende hoofdstukken naar voren komen.

In de biologische N-cyclus is de fixatie van stikstof uit de atmosfeer een sleutelreactie. Het enzym nitrogenase katalyseert de reductie van stikstof naar ammonia. De struktuur en functie van verschillende nitrogenases geïsoleerd uit verschillende organismen is zeer eender, dus de meeste conclusies die getrokken worden uit een onderzoek met één bepaald nitrogenase zijn ook op andere nitrogenases van toepassing. De in dit proefschrift beschreven experimenten zijn uitgevoerd met het nitrogenase uit Azotobacter vinelandii.

Het enzym wordt in vitro gemakkelijk gescheiden in twee componenten die beiden noodzakelijk zijn voor activiteit. De kleinste component wordt het Fe-protein genoemd en zijn functie lijkt beperkt te zijn tot het specifiek reduceren van de grotere component, het MoFe-protein. Deze electronenoverdracht gebeurt alleen als MgATP aanwezig is dat gehydrolyseerd wordt tot MgADP en Pi gedurende electronoverdracht. De uiteindelijke reactie, de reductie van stikstof tot ammonia vindt plaats op het MoFe-protein.

Beide eiwitten hebben ijzer/zwavel clusters, structuren die bestaan uit afwisselend ijzer en zwavel atomen, waar de benodigde electronen opgeslagen kunnen worden. Het Fe-protein wordt geacht slechts één ijzer/zwavel cluster van het [4Fe-4S] type te hebben, zoals dat ook gevonden is in ferredoxines. Een dergelijk type cluster kan slechts één electron opnemen of afstaan.

Ofschoon de experimenten die tot deze hypothese geleid hebben zeer overtuigend lijken, zijn er ook waarnemingen in de literatuur beschreven die niet verenigbaar zijn met het idee van één [4Fe-4S] cluster per molecuul Fe-protein en een één electron donor/acceptor gedrag in redox reacties.

Deze tegenstelling is ook in dit proefschrift te vinden. Bijvoorbeeld in hoofdstuk II worden redox reacties beschreven waarbij de redox toestand wordt gevolgd door middel van EPR spectroscopie en door activiteitsmetingen van het intacte enzym complex. Uit de hierbij verkregen resultaten kan geconcludeerd worden dat mogelijk twee electronen betrokken zijn in de redox reactie van het Fe-protein. De beschreven experimenten in hoofdstuk III bevestigen deze opvatting. In deze experimenten is het aantal electronen bepaald dat geoxideerd Fe-protein kan opnemen. In stopped-flow experimenten kon door kleurstof geoxideerd Fe-protein twee electronen opnemen van gereduceerd methyl viologeen. Daar het mogelijk is dat het Fe-protein te ver geoxideerd wordt door kleurstoffen is ook fysiologische oxidatie door MoFe-protein toegepast. Ook in dit geval zijn de verkregen resultaten meer in overeenstemming met de zienswijze dat Fe-protein zich gedraagt als een twee electronen donor/acceptor.

Aangezien een [4Fe-4S] cluster zich alleen als een één electron redox centrum kan gedragen onderzochten wij onze Fe-protein preparaten door middel van Proton Induced X-ray Emission Spectroscopy (PIXES) op de aanwezigheid van andere metaalionen die zouden kunnen functioneren als een electronenacceptor. Geen andere metalen buiten ijzer konden gedetecteerd worden. Maar we vonden een veel hoger ijzergehalte dan verwacht kon worden bij aanname van één [4Fe-4S] cluster.

Dit gaf aanleiding tot een grondige analyse van het ijzer-zwavel gehalte van Fe-protein (Hoofdstuk IV). Het maximum aantal ijzer en zwavel atomen dat gevonden werd per molecuul was acht.

Dit gecombineerd met de resultaten van Mössbauer experimenten die aangaven dat alle ijzer atomen zich in eenzelfde electronische omgeving bevinden, gaf aanleiding tot de veronderstelling dat mogelijk twee [4Fe-4S] clusters aanwezig zijn in Fe-protein.

In hoofdstuk V wordt aangetoond dat de groei condities het ijzer/sulfide gehalte bepalen van het geïsoleerde Fe-protein. Gedurende dit onderzoek was het maximum aantal ijzer en sulfide atomen per molecuul Fe-protein zes en niet acht. De tegenstelling tussen de waarden vermeld in hoofdstuk IV en V wordt bediscussieerd in de discussie van hoofdstuk V.

Mössbauer experimenten zijn niet beperkt gebleven tot het Fe-protein, maar ook het MoFe-protein is onderwerp van onderzoek geweest. Het belang

van onze resultaten is niet alleen beperkt gebleven tot het feit dat de specifieke activiteit van het gebruikte eiwitmonster veel hoger is dan bij anderen, maar ook de verwerking van de primaire gegevens is gewijzigd. Dit resulteerde in een andere verhouding ijzer/molybdeen van de FeMo-cofactor van MoFe-protein. Door dit feit is de samenstelling van de andere ijzer/zwavel clusters aanwezig in MoFe-protein nu twijfelachtig geworden.

Het is duidelijk dat het werk, beschreven in dit proefschrift, het probleem van de samenstelling en structuur van de ijzer-sulfide clusters in de nitrogenase eiwitten niet heeft opgelost. Buiten de beschreven experimenten, die zouden kunnen leiden tot de ontdekking van de ware aard van de clusters, is het aan het wankelen brengen van het algemeen aanvaarde dogma dat de structuur van de metaal-sulfide clusters is opgelost een ander belangrijk aspect van dit proefschrift. Dit heeft onderzoekers geprikkeld om opnieuw kritische experimenten uit te voeren om tot een beter begrip van de structuur en de functies van de clusters in nitrogenase te komen.

CURRICULUM VITAE

Arnold Braaksma is geboren op 24 september 1951 te Haarlem. Van 1964 tot 1968 doorliep hij de Klaas de Vries school en behaalde het MULO-b diploma. Vervolgens behaalde hij in 1970 het HBS-b diploma aan het toenmalig Laurens Coster Lyceum eveneens te Haarlem. Het daarop volgende jaar bracht hij onder 's lands wapenen door. In 1971 werd een studie biologie begonnen aan de Universiteit van Amsterdam. In 1974 werd het kandidaatsexamen B1 behaald en in 1979 werd de studie afgesloten met het doctoraal examen, met als hoofdvak Plantenfysiologie en bijvakken Bijzondere Plantkunde en Biochemie.
Van 1980 tot 1984 was hij werkzaam als promotie-medewerker aan de afdeling Biochemie van de Landbouwhogeschool te Wageningen in dienst van Z.W.O.
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