ASPECTS OF THE OXYGEN-TOLERANCE OF NITROGEN FIXATION IN *AZOTOBACTER VINELANDII*

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Proefschrift

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.LINGEN

Men moet de waarheid niet altijd openlijk uitspreken, en het is van groot belang hóe zij wordt uitgesproken. (Erasmus)

Niet alleen de plantenfysiologie, maar ook de plantenbiochemie en plantenziektenkunde zouden meer profijt kunnen en moeten trekken van reeds beschikbare of te selekteren mutanten. Mogelijkheden daartoe zijn sterk toegenomen door de ontwikkeling van cel- en weefselkweek van planten.

- Braaksma, F.J. (1982). Genetic Control of Nitrate Reduction in *Arabidopsis thaliana*. Proefschrift R.U. Groningen, Groningen, stelling 4.
- Chaleff, R.S. (1981). Genetics of Higher Plants. Cambridge University Press, Cambridge, pp. 184.
- Scherings, G. (1983). In Vitro Technieken bij de Induktie en Selektie van Somatische Varianten -Betekenis voor de Veredeling van Planten anno 1982. IVT Rapport 179. IVT, Wageningen, pp. 104.
- Scherings, G. en J.M. van Tuyl (1983). Gewasbescherming 14: 181-192.
- Somerville, C.R. (1982). What's New in Plant Physiology 13: 29-32.

Het wordt in het algemeen te weinig onderkend dat toepassing van sommige *in vitro* technieken bij de veredeling van generatief vermeerderde gewassen problemen met zich mee zal brengen, die analoog zijn aan die van de konventionele mutatieveredeling. Het feit dat nu toepassing van *in vitro* technieken wordt geëntameerd zonder dat relevante expertise in de konventionele mutatieveredeling voorhanden is of wordt nagestreefd, duidt op een weinig consistent onderzoeksbeleid.

- Anonyme (1983). Tissue Culture Techniques in Plant Breeding, an EEC Workshop. The plant Breeding Institute, Cambridge, pp. 14.
- Melchers, G. (1980) in: Perspectives in Plant Cell and Tissue Culture. Int. Rev. Cytol., suppl. 11B. Academic Press, New York, pp. 241-253.
- Scherings, G. (1983). In Vitro Technieken bij de Induktie en Selektie van Somatische Varianten -Betekenis voor de Veredeling van Planten anno 1982. IVT rapport 179. IVT, Wageningen, pp. 53-54.

Één bottleneck voor de toepasbaarheid van genetische manipulatie (in brede zin) bij kultuurplanten is de slechte regenerabiliteit van protoplasten van belangrijke kultuurgewassen. Bij het verwijderen van die flessehals lijkt studie van de fundamentele processen die regenerabiliteit bepalen op fysiologisch/biochemisch nivo een rationelere aanpak dan het doorlichten van komplete genenbanken op erfelijke regenerabiliteit.

- Boss, W.F., N.S. Allen and H.D. Grimes (1983). in: Protoplast1983 Poster Proceedings (Potrykus, I., C.T. Harms, A. Hinnen, R. Hütter, P.J King and R.D. Shillito, eds.). Birkhäuser Verlag, Basel, pp. 96-97.
- Hock, K. (1983). Ibid. pp. 206-207.
- Potrykus, I. and J. Petruska (1983). Ibid. pp. 12-13.

De *in vitro* selektieprocedure van Thomas en Pratt op paraquat-resistentie in tomaat gaat voorbij aan het bekende mechanisme van paraquat-toxiciteit in planten en is landbouwkundig irrelevant. Hetzelfde geldt voor een vergelijkbare selektie van Miller en Hughes in tabak.

- Chia, L.S., D.G. McRae and J.E. Thompson (1982). Physiol.Plant. 56: 492-499.
- Miller, O.K. and K.W. Hughes (1981). In Vitro 16: 1085-1091.
- Thomas, B.R. and D. Pratt (1982). Theor.Appl.Genet. 63: 169-176.

5. De suggestie van Maris et al. dat Azotobacter cellen ook in aanwezigheid van NH_4^+ een eiwit synthetiseren dat immunologisch niet te onderscheiden is van nitrogenase is misleidend. De auteurs gebruikten een niet-specifiek antiserum waarvan zelfs niet is aangetoond dat het met *gezuiverd* nitrogenase reageert.

   - Maris, D.J., Li-Wen Wang, P.B. New and Y.T. Chan (1983). in: 5th Symposium on Nitrogen Fixation, Book of Abstracts, abstract 4B2.

6. De verhouding van de opbrengst van gereduceerd produkt tot de ingestraalde hoeveelheid lichtenergie in artificiële fotosystemen met tertiaire aminen of thiol-verbindingen als electronendonor wordt meestal overschat aangezien deze veelgebruikte donoren na oxidatie ook in een donkerreaktie gereduceerd produkt kunnen leveren.

   - Krasna, A.I. (1980). Photochem.Photobiol. 31: 75-82.
   - Rougee, M., T. Ebbesen, F. Ghetti and R.V. Bensasson (1982). J. Phys.Chem. 86: 4404-4412.

7. Door de huidige situatie: een tweedeling van aan de ene kant langdurig werkloze en aan de andere kant hooggesalarieerde maar overbelaste academici, te laten voortbestaan, maakt de overheid zich schuldig aan vernietiging van kapitaal en kreativiteit op grote schaal.

8. Bepaalde vormen van diskriminatie kunnen van belang zijn voor de sociale zelfbevestiging van het individu en zijn derhalve, in strikt gekontroleerde vorm, niet *a priori* te veroordelen.

9. De huidige trend om ook bij sollicitatieprocedures voor *wetenschappelijke* funkties het begrip 'contactuele eigenschappen' als een belangrijk selektiekriterium te hanteren dient, zeker door universitaire instellingen, met gepaste reserve te worden bezien.

10. De nadruk die christendemocraten plegen te leggen op het gezin als hoeksteen van de samenleving zou, indien hij is bedoeld als waarborg voor instandhouding van de eigen ideologie, gezien het grote aantal PvdA-kopstukken met confessionele achtergrond wel eens averechts kunnen werken.

G.H. Scherings
Aspects of the oxygen-tolerance of nitrogen fixation in *Azotobacter vinelandii*
december 1983, Wageningen.

'S-slaagsysteem 101A?' vroeg heer Ollie vol bange voorgevoelens. 'W-wat is dat voor iets?' 'Dat is de methode om door *kennis* iets te bereiken, legde de onderwijzer uit. 'Ik geef toe dat het ver gezocht is - maar nog-maals, we hebben geen keus!'

Marten Toonder, Het slaagsysteem. in: Zeg nu Zelf.....De Bezige Bij, Amsterdam, 1974, pp. 205.

Een mens moet eeuwig zijn keuze doen tussen kreatieve uitingen, 'the struggle for life' en de zich voordoende mogelijkheden van volvoerende ontdekkingen.

Ben Borgart, De vuilnisroos. De Bezige Bij, Amsterdam, 1972, pp. 149.

Aan mijn ouders

VOORWOORD

Sinds de dagen van Justus Liebig (1803-1873) en Jean-Baptiste Boussingault (1802-1887) hebben landbouwkundigen de letter *N* tot één van de belangrijkste uit het landbouw-alfabet gerekend. Het aantal veldproeven waarin het effect van N (naast P en K) op de opbrengst van een gewas werd bekeken moet intussen van een welhaast astronomische orde zijn.

De stikstofvoorziening van landbouwgronden is dus al sinds het begin van de landbouwwetenschap een 'hot issue'. Tegenwoordig is N een *sine qua non* voor de *status quo* van de voedselvoorziening. *N 'moet'*.
Zoals bekend heeft het nivo van stikstofbemesting in Nederland en andere rijke landen reeds enige tijd het gebied van de afnemende meeropbrengsten bereikt. Het is echter goed te beseffen dat de overvloed aan voedsel die voor een groot gedeelte daaraan te danken is, niet anders is dan 'living on borrowed time': dansen op de vulkaan. En wel, omdat ze is gebaseerd op enerzijds verbruik van 'non-renewable energy sources', anderzijds een hoogst merkwaardige distributie van ekonomische en politieke macht tussen rijke en arme landen. Aan het laatste kan de wetenschap helaas niets direkt veranderen; wél aan het eerste.
Nog steeds worden vrijwel alle stikstofmeststoffen industrieel geproduceerd op basis van het Haber-Bosch-procédé. Op zich is dit een zeer efficiënt procédé, gegeven de beide grondstoffen N_2 en H_2. N_2 is inderdaad 'gegeven', d.w.z. bijna voor niets uit de atmosfeer voor verwerking beschikbaar. De produktie van H_2 zoals die thans geschiedt, voornamelijk uit CH_4 (aardgas), is daarentegen energie- en grondstof-verslindend. Eén strategie om goedkopere stikstofmeststoffen te produceren is daarom het zoeken naar efficiëntere manieren om H_2 te produceren, en bij voorkeur niet uit een schaars goed als aardgas.
Andere strategieën zijn, te proberen de 'natuurlijke', biologische, wijze om luchtstikstof te binden biotechnologisch hanteerbaar te maken, dan wel chemisch na te bootsen, dan wel de erfelijke eigenschappen die dit proces bepalen, in te bouwen in kultuurgewassen. Het is de wens om deze laatste strategieën te realiseren die mede ten grondslag ligt aan de grote vlucht die het genetische, fysiologische en (bio)chemische onderzoek aan de biologische stikstofbinding sinds twee decennia heeft genomen.
Ik dank mijn promotor, prof.dr. Cees Veeger, van harte dat hij mij de gelegenheid heeft gegeven een klein steentje bij te dragen aan dit dynamische onderzoeksterrein.
Ik dank de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek (ZWO)

voor financiële ondersteuning daarbij.

I would like to extend my gratitude to the ARC Unit of Nitrogen Fixation at the University of Sussex, Brighton, for their hospitality during the summer of 1977. I am particularly indebted to Dr. M.G. Yates and Mrs. Frances Campbell, who taught me how to handle nitrogenase 'with care'.

Het is natuurlijk niet zo dat voor het dragen van wetenschappelijke steentjes bijzondere, zwaardere, gravitatiewetten gelden dan voor alledaagse. Dit boekje is de neerslag van vier jaren werk, niet meer, niet minder. Er waren vele *ups*, en evenzovele *downs*; soms was het werk prettig, soms ook minder prettig. Kortom, gewóón werk. De gewoonte om in het voorwoord bij een proefschrift vrienden, bekenden en labgenoten uitbundig lof toe te zwaaien voor bewezen diensten, als was men zojuist aan een ware Sisyphus-arbeid ontsnapt, en dat dan nog slechts dankzij een laatste krachtsinspanning en dankzij talrijke toegestoken handen, komt mij dan ook weliswaar begrijpelijk, doch enigszins overdadig voor. Toch moet ik voor één persoon een uitzondering maken, en wel voor mijn co-referent, Dr. Huub Haaker. Dat heeft alles te maken met de enigszins ongewone omstandigheden waaronder dit proefschrift tot stand is gekomen. Dat dit werk, vier jaren na feitelijke beëindiging ervan, met dit geschrift kan worden afgesloten op de naar geldende inzichten passende wijze, is aan hem te danken. Het vertrouwen dat hij in mij heeft gesteld, gevoegd bij zijn persoonlijke en wetenschappelijke integriteit en tegelijkertijd informaliteit die vier jaar lang voor een prettige samenwerking borg hebben gestaan, rechtvaardigt een 'Huub, bedankt!' uit de grond van mijn hart.

CONTENTS

I. GENERAL INTRODUCTION

A striking contrast exists between two main chemical components of the biosphere, N and C, in the way they are incorporated from inorganic precursors. Whereas the precursor of organic C, CO_2, is present only in near-trace amounts in the atmosphere, the precursor of organic N, N_2, actually is the main constituent of the atmosphere. Whereas C is incorporated by a widely, in prokaryotes as well as in eukaryotes, distributed process, called CO_2 fixation or 'Calvin cycle', the process for incorporating N called N_2 fixation, is very sparsely distributed, and exclusively in prokaryotes (123). Why there should be such an unequal distribution of these two processes, both of prime importance to life, is an interesting question for students of evolution.

It is generally accepted that without either a major expansion of industrial nitrogen fixation, or the development of alternative technologies for increasing biological nitrogen fixation, the available nitrogen will continue to be the most important single factor limiting world agricultural productivity (62). Expansion of industrial fixation would probably rely heavily on 'non-renewable energy sources' and is therefore in the long run not feasible; development of alternative technologies seems far more attractive as a long-term investment.

It is therefore somewhat surprising that, till recent times, the study of biological nitrogen fixation has been neglected, at least far less intensively studied than photosynthesis. Thanks to the energy crisis, priorities have changed*. Several laboratories have been established worldwide that have the study of nitrogen fixation as their main or exclusive research aims. Among the forerunners were (and still are) the Charles F. Kettering Laboratories at Yellow Springs, where N_2 fixation was designated a major research target along with photosynthesis, and the ARC Unit of Nitrogen Fixation at Brighton with N_2 fixation as sole and exclusive research subject. It seems quite appropriate that nitrogen fixation nowadays should be a booming area for study, at all levels, from agriculture to genetics, physiology, and (bio)chemistry. Recent progress in these separate fields has been monitored and discussed in the reviews mentioned below. Besides, a monograph encompassing virtually all these fields in summary has appeared recently (108a).

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*One doesn't have to go back far to find a historical parallel. In 1893, Sir William Crookes warned the British Association for the Advancement of Science that the nitrate deposits of Chile, till then the major source of fixed nitrogen for agriculture (and warfare, incidentally), were approaching depletion (13). Twenty years later, the first commercial ammonia manufacturing plant was established in Germany, based on the now-famous process patented by Fritz Haber in 1908 (54).

agriculture	:	(61)
genetics	:	(3,116,69)
physiology	:	(143,71,124,112,33)
biochemistry	:	(36,57,91)
chemistry	:	(59)

Biochemical studies of nitrogen fixation are mainly concerned with the enzyme that 'fixes' N_2, called *nitrogenase*. One outstanding difficulty with this complex enzyme is its extreme sensitivity to oxygen. This property has frustrated many early studies; moreover, it is exactly this property of nitrogenase that will predictably frustrate attempts to genetically 'engineer' nitrogen fixing crop plants (13,1,84) unless proper attention is paid to it. So far, the oxygen sensitivity of nitrogenase hasn't received the attention it thus deserves, but interest does seem to quicken (42,105). Hopefully, this thesis will contribute to that by focusing on the nitrogenase of that most aerophilic (or perhaps least aerophobic) of nitrogen fixing organisms, *Azotobacter vinelandii*.

I.1 Nitrogenase

Nitrogenase proteins, i.e. enzymes that are able to physically bind N_2 and mediate its reduction to NH_3, are remarkably similar, whether they are obtained from aerobic or anaerobic, symbiotic or free-living organisms. Therefore many properties of nitrogenases can be discussed without making reference to their origin. If reference is made, however, it usually occurs in an abbreviated way, as follows: nitrogenase from *Azotobacter vinelandii* consists of Av_1 and Av_2; that from *Clostridium pasteurianum* of Cp_1 and Cp_2; that from *Rhizobium japonicum* of Rj_1 and Rj_2. In this nomenclature, originally proposed by the Brighton group (38), it is implicit that a nitrogenase enzyme consists of two proteins, namely the components 1 and 2. Both are required for activity.

Component 1, also referred to as *FeMo protein*, is a large tetrameric ($\alpha_2\beta_2$) protein (M_r 220-245 kDa). It contains 2 molybdenum, 28-32 iron, and about 28 acid-labile sulfur atoms (36). The role of these atoms in catalysis is not yet clear but is under continuous investigation (17,99). About half of the number of Fe atoms seems to be organised into two iron-molybdenym cofactors, one per $\alpha\beta$-unit (36). These cofactors, called FeMoCo, probably contain the binding site(s) for N_2 (119). The remaining Fe is probably organised into four [4Fe-4S] clusters (36), the so-called P-centres.

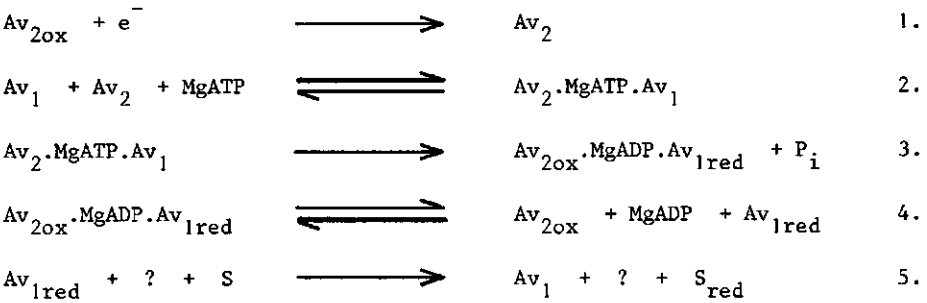
Interestingly, FeMoCo appears to be related to another Mo-cofactor from an enzyme involved in nitrogen metabolism, nitrate reductase. The latter cofactor

has been called MoCo (102). In a *nar-D* mutant of *Escherichia coli*, defective in MoCo synthesis (or perhaps Mo-mobilization), Kp_1 polypeptides may be synthesized from a full complement of plasmid-borne *nif*-genes from *Klebsiella pneumoniae*, - however not a functional Kp_1 holoprotein. In wild type *E.coli* on the contrary a fully functional Kp_1 holoprotein *is* synthesized (70). Thus, in cells that contain both *nar*- and *nif*-genes, MoCo may be a precursor for FeMoCo (36). Both MoCo and FeMoCo can be isolated from their respective apoproteins by precipitation and extraction with organic solvents (17,106). Isolated FeMoCo is extremely sensitive to oxygen, this in contrast to the FeMo holoprotein which is only moderately so (36). A last property of the FeMo protein which should be mentioned is that it may contain binding sites for MgATP. At least four weak binding sites have been demonstrated in Kp_1 (89); however, in Av_1 no binding sites at all could be demonstrated (24). Whether such binding sites have relevance to nitrogenase activity is not clear.

Component 2, also denoted as *Fe protein*, is a smaller, dimeric (γ_2) protein, M_r 60-65 kDa (36). As isolated, it usually contains 4 iron and about 4 acid-labile sulfur atoms, organised into an [4Fe-4S] cluster (43,97). However, recent findings indicate that there may be other possibilities: fully active Av_2 protein probably contains at least eight Fe atoms (12). Fe protein is much more sensitive to oxygen than FeMo protein; quoted half lives in air range from 2 min to 45 sec (37). Fe protein contains binding sites for MgATP and MgADP. Especially binding of MgATP alters the physical properties of reduced Fe protein, as evidenced by: (a) increased reactivity of Fe and sulphydryl towards specific reagents, (b) increased sensitivity to oxygen, (c) a significantly lowered midpoint redox potential and (d) a change in the form of the e.p.r.-signal (36,37). These effects are usually interpreted as indicating a gross effect of MgATP on protein tertiary structure, especially around the [4Fe-4S] cluster (36). The *number* of binding sites for MgATP and the characteristics of MgADP *vs.* MgATP binding were for some time thought to be well established: two sites for MgATP binding and competitive binding of MgADP *vs.* MgATP (36,91). However, more recent results of Cordewener *et al.* (24) indicate that some of the previous work may be in error, due to insufficient attention to redox state and integrity of the Fe protein, both of which may change considerably in the time course of conventional (time consuming) measurements. The careful experiments of Cordewener *et al.* (24) indicate that free, reduced Av_2 can bind only one molecule of MgATP. If, as Hageman *et al.* (58) propose, two molecules of MgATP must bind to Av_2 before electron transfer to Av_1 can take place (see below), the second binding site needs to be *generated* after binding of Av_2 to Av_1. -If anything, it should be

clear that the Fe protein, though simpler than the FeMo protein, is still not an open book.

The two nitrogenase components interact with each other during catalysis; however, the nature and extent of this interaction have not been established definitively yet and are the subject of some controversy. Still, at least a general sequence of events has been accepted by most for the time being, as follows (Scheme 1).



Scheme 1. Tentative sequence of events in nitrogenase catalysis. For clarity, the abbreviated denotation for the Fe and FeMo proteins from *A.vinelandii* has been used, though the scheme is meant to represent the functioning of nitrogenase generally, irrespective of the source. No effort has been made to represent the reactions stoichiometrically. Possibly two molecules of MgATP have to bind before inter-protein electron transport (step 3) can take place (58). If Av_2 accommodates only one reducing equivalent (step 1), at least two cycles of steps 2-4 will be necessary before step 5 can take place. After Mortenson and Thorneley (91).

It is clear that the central issue in nitrogenase function, as its formal name 'reduced carrier: dinitrogen oxidoreductase (ATP-hydrolyzing)' (EC 1.18.2.1) indicates, is *electron transfer* to the final substrate N_2. Although extensive studies have been performed on the redox properties of the Fe and FeMo proteins (36,81,91,120,147) there are still gaps in our knowledge (11). One reason for this is, again, the oxygen sensitivity of these proteins, which specifically may affect the redox centers (37); due to this it is quite hard to obtain intact (fully active) protein. Owing to these and other problems, it would be virtually impossible to define stoichiometries of the reactions that are, roughly, outlined in Scheme 1. For further comments on the crucial importance of establishing the degree of integrity of any nitrogenase preparation in the evaluation of enzymological studies, see the Discussion in Hageman *et al.* (58).
The experimental data on each of the steps 1-5 have been reviewed by Mortenson

and Thorneley (91). Steps 1 and 2, reduction of the Fe protein and subsequent complex formation of the reduced Fe and FeMo proteins and binding of MgATP, are relatively little debated. Several questions may be raised , however. For instance, step 1, though it is often represented as an irreversible reaction (91,131), is a truly reversible reaction, at least with artificial electron donors such as dithionite (11). In step 2, the sequence of binding of MgATP and of Av_2 to Av_1 relative to each other is questionable (11). About step 3, all that can be said with certainty is that electron transfer from the Fe protein to the FeMo protein is dependent on the presence of MgATP and is somehow coupled to the hydrolysis of ATP. Because the demand for ATP determines to a large extent the efficiency of the overall process, this will be discussed further below (I.2.2). In any case, starting from the dithionite-reduced state, step 3 results in the formation of a 'super reduced' state of the FeMo protein.

It should be made clear that the term 'superreduced state' by itself does not mean a great deal; according to the hypothetical scheme of Thorneley and Lowe (130,131) it is just one of the redox states that the FeMo protein may assume in a catalytic cycle. Similarly, while sometimes the dithionite-reduced state is regarded as the normal 'resting state' (131), this does not mean that it necessarily has any special significance in catalysis; on the contrary, the specific e.p.r. signals that are exhibited by the FeMo protein in the dithionite-reduced state are not usually observed during turnover.

What happens after step 3 is hotly debated. Step 4 as given indicates that the complex dissociates after each $1e^-$-transfer, as deduced by Hageman and Burris (56) and generally accepted (91,131). However, one should remain conscious of the fact that the deductions of Hageman and Burris (56) were based on kinetic studies on the hydrogen evolution reaction of nitrogenase in which (a) the reaction mechanism may be quite different from the physiologically more important N_2 reduction reaction (131), and (b) the concentrations of enzyme are much lower than would be the case *in vivo* (65,111). Further evidence that the proposal of Hageman and Burris (56) may relate to an arteficial *in vitro* situation only can be drawn from studies by Braaksma *et al.* (11,12) indicating that *intact* Fe protein may accommodate two reducing equivalents which perhaps are transferred simultaneously to the FeMo protein.

The last step of Scheme 1 is hottest debated. Concerning this step, Burris and his coworkers have repeatedly argued (19,58) that FeMo protein, at a suitable redox level, is able to reduce substrates on its own, without further involvement of the Fe protein. Their scheme (Fig. 1) has the virtue of simplicity but many authors regard it as an oversimplification (126,131). In Burris' view, the

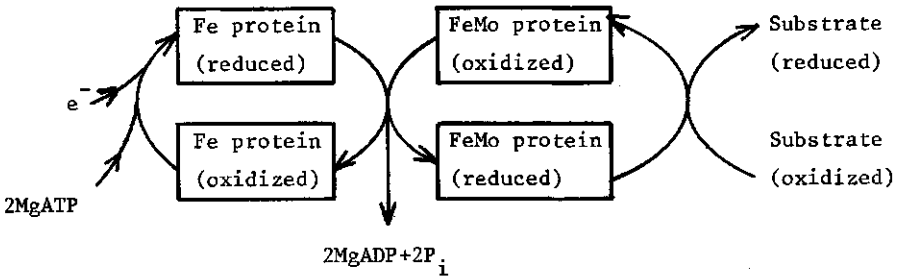


Fig. 1. A simplified scheme outlining electron flow and ATPase activity in nitrogenase. In this scheme, e^- indicates a low potential electron donor: *in vivo* usually a flavodoxin or ferredoxin (46,143); *in vitro* almost always dithionite ($SO_2^{\cdot -}$). Substrate may of course be N_2 but also a host of other simple compounds such as N_2O or CN (if present) or - last but not least - H^+ (which of course is always present). H^+ reduction is probably mechanistically different from the reduction of other substrates since (a) all other substrates contain triple bonds and (b) H^+ reduction is not inhibited by CO whereas reduction of all other substrates is. In routine *in vitro* activity measurements C_2H_2 is employed as the reducible substrate because only one product is formed (C_2H_4) which can be very easily and accurately measured.
Figure from Stewart (123) after Burris *et al.* (19).

Fe protein acts exclusively, though very specifically, as an electron pump which reduces the FeMo protein. In accordance with that idea, he proposed to rename the FeMo protein as *'dinitrogenase'* (the 'true' nitrogenase), the Fe protein as *'dinitrogenase reductase'* (19). For the time being, this proposal has been rejected (126), because other evidence and considerations point to a probability that the Fe protein has more than one role to fulfill, specifically in preventing electrons from being allocated to H^+ instead of N_2 (131). According to Thorneley and Lowe (131), further interactions of Fe protein with FeMo protein are desined to the effect that the turnover of nitrogenase is effectively slowed down to a rate where the level of reduction of Mo can be kept sufficiently low to allow efficient competition of N_2 relative to H^+ or H_2. This would fit in nicely with considerations based on chemical model studies (23). -The uncertainty about step 5 in Scheme I has thus been indicated by a question mark; it may well be that a complex of the Fe and FeMo proteins, rather than the FeMo protein alone, is involved in substrate reductions other than those of H^+. One reason that really unequivocal experiments concerning this debate are presently difficult or impossible to design is the lack of inhibitors at the reducible substrate side: once electron transfer between Fe protein and FeMo protein has taken place, subsequent transfer to substrate cannot be stopped or in-

hibited. If N_2 as a substrate is omitted from the reaction mixture, H^+ will take its place as an alternative substrate. Indeed, up to now it appears physically impossible to isolate the FeMo protein in a 'superreduced' state or any form that will reduce substrate by itself (131). Physical studies on 'superreduced' FeMo protein *are* possible, though, by employing extreme conditions such as low temperature (2-4°K) plus a high ratio of FeMo/Fe protein (122).

I.2 Integration of nitrogenase into cellular metabolism

I.2.1 On the price of nitrogen fixation

In vitro, the demands for nitrogenase action are, at least qualitatively, easily enough met. Dithionite can be used as a ready supply of low-potential electrons, and at the same time scavenges residual oxygen from the reaction mixture. The supply of (Mg)ATP can be ensured by an ATP regenerating system (usually creatine phosphate/creatine kinase), which simultaneously keeps the level of (Mg) ADP, a powerful inhibitor, at a minimal level.

For the experimenter, the economy or efficiency of such a reaction is not usually a (high) priority. However, if 'survival of the fittest' is the law, of course things are different. Indeed, the low frequency with which the trait 'diazotrophy' is encountered in Nature (123) may well be an indication of some high price that has to be paid for being able to carry such an, obviously otherwise extremely useful, trait (15). Other indications that diazotrophy does carry a considerable price tag may be found in the following facts, from genetic (a) and metabolic (b) studies.

(a) The expression of the 'diazotrophy' trait appears to be subject to strict regulation. In *K.pneumoniae*, the trait was exclusively expressed in the absence of oxygen and severely repressed in the presence of any source of fixed nitrogen, especially NH_4^+ (103,127). In fact, it has been stated that 'the repressive effect of NH_4^+ is several orders of magnitude greater than that usually found for other enzyme systems' (14).

(b) The 'energy charge' (2) of *C.pasteurianum*, *K.pneumoniae* and *A.vinelandii* (respectively obligate anaerobic, facultative aerobic, and obligate aerobic organisms) declined in all three cases by > 15% when sucrose-limited cells were shifted from N_2-dependent to NH_4^+-dependent growth (132). 'Energy charge' may be regarded as a measure of metabolically available energy, defined as a ratio of adenine nucleotides: (ATP + 0.5 ADP)/(ATP + ADP + AMP). Since these experiments by Upchurch and Mortenson (132) were, however, performed mainly on *batch* cultures, other factors may have played a confusing role, as indeed has

been shown by the experiments of Marriott *et al.* (85) on N_2-grown *chemostat* cultures of *A.beijerinckii*. Whether 'energy charge' still can be considered a useful concept in studies of the energy requirements of nitrogen fixation remains to be established. A critical review of some early data has been given by Postgate *et al.* (109).

The concept of 'energy charge' should in any case not be considered a universal indicator of the energy status of any type of cell. At least in cells that synthesize most of their ATP at membrane-bound H^+-translocating MgATPases (i.e. respiring or photosynthetic cells), the concept of 'membrane energy' or *protonmotive force* may be more useful. In a series of elegant papers by the Veeger group (48,52,76,78,79) it has been convincingly shown that small (artificially induced) changes in the protonmotive force, 'energy charge' constant, correlate well with quite dramatic changes in nitrogenase activity, both in *A.vinelandii* and *R.leguminosarum*. Thus, in these organisms even at relatively high 'energy charge', nitrogenase activity is subject to regulatory mechanisms related to the energy metabolism of the cell. Indications that such mechanisms exist in photosynthetic nitrogen fixers as well have been found with the cyanobacterium *Anabaena variabilis* (64) and the photosynthetic bacterium *Rhodopseudomonas sphaeroides* (49).

Despite evidence for N_2 fixation being a major 'energy drain' in cellular metabolism and (therefore) being under tight control, still a precise calculation of the 'cost' of biological nitrogen fixation cannot be given. Indeed, this should be a major worry for people anxious to 'engineer' nitrogen fixing crop plants: the possibility cannot be dismissed yet that the cost of crop diazotrophy will equal or exceed that of manufacturing, storing and distributing industrially fixed nitrogen. Only recently, research recommendations have been given in order to fill that gap in our knowledge (115).

A *minimum* price of diazotrophy however, may be deduced from the following reaction formula which is generally accepted to represent the substrate and product stoichiometry of nitrogenase under optimum conditions (123):

$$N_2 + 10H^+ + 8e^- + 16ATP \xrightarrow[N_2ase]{Mg^{2+},\ \text{anaerobiosis}} 2\ NH_4^+ + H_2^* + 16\ ADP + 16\ P_i \quad \text{(Eq.1)}$$

*The evolvement of H_2 is probably an unavoidable consequence of the mechanism of binding of N_2 to the active site of nitrogenase, namely, by displacement of H_2 (131). Studies of model systems support this concept (114,23). This may be specific for N_2 as the substrate: in C_2H_2 reduction, the product is almost exclusively C_2H_4 (60).

In the following, two components of this minimum cost will be summarily discussed: energy requirement of nitrogenase (I.2.2) and maintenance of anaerobiosis (I.2.3).

I.2.2 Energy requirement of nitrogenase

Nitrogenase complexes isolated from *A.vinelandii* and *Chromatinum vinosum* with electrochemically reduced viologens or dithionite as electron donors show full activity at redox potentials below -500 mV and no significant activity at redox potentials higher than -430 mV (40,135). In Chapter II of this thesis it will be shown that, using flavodoxin from *A.vinelandii* as electron donor, a nitrogenase complex from the same organism is fully active at redox potentials below -500 mV but inactive at redox potentials above -460 mV (see also ref. 11). Braaksma *et al.*(11) found that, with photochemically reduced viologens, flavodoxin from *Megasphaera elsdenii*, or dithionite as electron donors, purified nitrogenase from *A.vinelandii* keeps functioning up to a redox potential of -350 mV, activity being maximal below -440 mV. Thus, despite the fact that the couple N_2/NH_4^+ is characterized by a relatively high midpoint potential ($E_{m,7}$ = -280 mV), the 'working potential' of nitrogenase is, physiologically speaking, quite low. In this sense nitrogenase is comparable only to such typically *anaerobic* enzyme reactions as CO_2 reductase ($E_{m,7}$ = -420 mV), hydrogenase ($E_{m,7}$ = -420 mV) or pyruvate:ferredoxin oxidoreductase ($E_{m,7}$ = -510 mV). This poses problems for *aerobic* nitrogen fixers, as will be discussed below.

The relatively low redox potential at which nitrogenase operates apparently calls for specialized redox carriers, in order to link the enzyme to the rest of cellular metabolism. So far, electron carriers of two physiological kinds have been shown to interact directly with nitrogenase, namely *flavodoxins* (soluble proteins, containing FMN as prosthetic group, with molecular weights ranging from 15-25 kDa) (86)) and *ferredoxins* (mostly soluble proteins, all containing Fe-S structures as prosthetic groups but otherwise rater inhomogeneous with respect to molecular properties (82)). The properties of these redox proteins have been reviewed quite adequately elsewhere (46,82,86). In most cases it is not clear whether either of the two types has specific advantages as electron donor for nitrogenase; in *K.pneumoniae* , however, it has been proven unequivocally by genetic analysis that *in vivo* flavodoxin, not ferredoxin, serves as electron donor for nitrogenase: the gene coding for flavodoxin is part of the *nif* gene cluster (10,95). In *A.vinelandii* and *A.chroococcum* circumstantial evidence points to flavodoxin being the physiological donor (this thesis, chapter II, and refs. 141,146). Though in *A.vinelandii* flavodoxin appears be present also in small amounts in NH_4^+-grown

cells, after derepression (transfer to N_2-dependent growth) synthesis of flavodoxin parallels synthesis of the nitrogenase structural proteins (Klugkist, pers.comm.) and therefore may be also (partially) *nif*-specific. In other organisms both types may be interchangeable, e.g. in *C.pasteurianum* (73), where one or the other may be synthesized, depending on growth conditions. -In any case, qualitatively speaking electron transport between nitrogenase and these types of carriers does not seem to pose any specific demands *in vitro* . The efficiency of electron transfer of course may vary considerably, depending on midpoint redox potential and other molecular properties of the carrier involved (cf. chapter II of this thesis). An interesting point in this context is the electron transfer between Av_2 and the so-called 'Shethna' protein of *A.vinelandii*. The latt is a ferredoxin of the type containing [2Fe-2S] clusters, that plays such an important role in photosynthetic electron transport (128). In chapter III of this thesis it is shown that electron transfer between these two Fe-S proteins is very efficient, which may have important consequences for the protection of Av_2 against inactivation by oxygen (see below, chapter I.2.3).

As hinted above, despite the fact that ferredoxins were actually discovered as a new class of electron carriers in the course of an investigation on nitrogen fixation in the anaerobe *C.pasteurianum* (92), both flavodoxins and ferredoxins are ubiquitous in anaerobic organisms and fulfill numerous functions apart from donating electrons to nitrogenase. In these organisms, they themselves accept electrons from pyruvate *via* the appropriate oxidoreductase. Although other e^--dono for nitrogenase have been suggested; H_2 (27) or formate (90), most probably these compounds should on the contrary physiologically be viewed as products, not substrates, of metabolism. After all, anaerobes are often characterized by excess production of reducing equivalents relative to ATP production and will somehow have to dispense with that excess (28,68,129). In short, nitrogen fixtion fits in quite easily on the map of *anaerobic* metabolism.

The remaining, real enigma in the integration of nitrogenase into cellular metabolism, both as far as generation of reducing equivalents (below) and maintenance of anaerobiosis (I.2.3) is concerned, lies with the obligate *aerobe* diazotrophs. Although in some of these, e.g. the cyanobacterial ones, the enzyme pyruvate:ferredoxin oxidoreductase has been found (94), activities *in vitro* are usually very low and certainly too low to account for the nitrogenase activities observed *in vivo* (46). The 'enigma' is that apparently nobody has been able so far to obtain satisfactory rates of nitrogenase activity in cell-free extracts of aerobe diazotrophs by just adding cofactors and those carbon substrates (glucose-6-phosphate, malate) that might be expected to be able

to generate enough reducing power to drive nitrogenase. Now and again reports appear in the literature claiming nitrogenase activity in cell-free extracts with 'physiological' e^- donors, e.g. with pyruvate in photosynthetic bacteria and cyanobacteria (83,94) or NADPH *plus* an efficient NADPH-regenerating system, e.g. in *A.vinelandii* (6). However, in all cases the reported rates are just a fraction of the activity obtained with dithionite as electron donor. The candidate which has been advocated most persistently as 'the' physiological electron donor for nitrogenase (*via* flavodoxin or ferredoxin) in aerobe diazotrophs is NAD(P)H. However, the reduced pyridine nucleotides, despite the fact that they are probably the most common currency of reducing equivalent in aerobes, are *a priori* somewhat unlikely candidates as indeed has been pointed out emphatically by Haaker *et al.* (46,50,133). Their reducing power is only moderate ($E_{m,7}$ = -320 mV)*, relative to the 'working midpoint potential' of nitrogenase of *at least* -400 mV (see above). However, NAD(P)H has still not been ruled out definitively since many factors probably influence the 'working potential' such as the ratio of Fe protein over FeMo protein, Mg^{2+}-, ATP- and ADP-concentrations, all of which may *in vivo* be vastly different from the usual *in vitro* conditions.

An interesting point in case is the argument put forward by Gutschick (44, 45). The argument essentially says that the irreversibility of the inter-protein e^--transport in nitrogenase, coupled to ATP hydrolysis (cf. chapter I.1) would keep the Fe protein ($E_{m,7}$ ranging from -290 mV for Cp_2 (147) to -390 mV for Av_2 (11)) sufficiently oxidized for NAD(P)H ($E_{m,7}$ = -320 mV) to be able to function as an efficient electron donor to the oxidized Fe protein (of course, *via* flavodoxin or ferredoxin and the appropriate oxidoreductase). Subsequently, MgATP would bind to the reduced Fe protein, transforming the redox potential to a value sufficiently low for the Fe protein to act as an efficient e^--donor to the MoFe protein and substrate. As Haaker (46) points out correctly, this presupposes that (a) the pools of NAD(P)H and flavodoxin/ferredoxin are in effective redox equilibrium via the appropriate oxidoreductase and (b) there is no redox equilibrium between Fe protein and Fe protein with MgATP bound. Neither supposition has been substantiated yet by experiment; the first supposition is problematic in any case since (a) efficient NAD(P)-ferredoxin (flavodoxin) oxidoreductases have rarely been convincingly demonstrated in aerobes (46) and moreover, are usually severely inhibited by NAD(P)H and (b) if such an equilibrium does exist, there would be no point for the cell in synthesizing such specialized

*That is, assuming that NAD(P)H acts exclusively as a $2e^-$-donor.

electron carrying proteins as flavodoxins with midpoint redox potentials as low as -495 mV (flavodoxin from *A.vinelandii* (5)). Further evidence against Gutschick's proposal is provided by the following two well-established facts:
(1) As stated above, nobody has ever been able to obtain reasonable rates of nitrogenase activity, either in cell-free extracts of aerobes with organic substrates capable of generating a high NAD(P)H/NAD(P)$^+$ ratio, or in artificial reaction mixtures with very efficient NAD(P)H-regenerating systems plus flavodoxin or ferredoxin plus an efficient oxidoreductase (46). Jungermann *et al.* (67), using a very efficient NAD(P)H regenerating system in combination with the aspecific electron carrier methyl viologen, found with nitrogenase from *A.vinelandii* only 10% of the dithionite activity, against 40% of dithionite activity with nitrogenase from *C.pasteurianum*.
(2) Measurements of the specific e.p.r. signal from the Fe protein during nitrogenase turnover (93,98,121) have shown that then it is at most for 50% in the oxidized state; and certainly not oxidized to the extent that Gutschick's proposal would require.
The one attractive feature of Gutschick's proposal (44,45), however, is that it offers an (admittedly teleological) explanation for the ATP requirement of nitrogenase. Otherwise, the question why ATP should be hydrolysed during nitrogenase turnover remains somewhat of a mystery since, after all, the reduction of N_2 to NH_3 is an exothermic one (113); the argument of Gutschick's leaves open the possibility that ATP hydrolysis should be coupled to electron transfer *in order to 'pull'* electrons against a redox gradient. This should be contrasted to the proposal of Haaker (46) based on data from Jungermann *et al.* (67) for anaerobic diazotrophs, where reducing equivalents from NADH might by 'pushed' towards ferredoxin (and thus nitrogenase) through coupling to the hydrolysis of Ac∿CoA. A similar but more complex proposal has been put forward by Haaker *et al.* (46,133) and Laane (75) for obligate aerobe diazotrophs, where electrons from NADPH might be pushed towards flavodoxin (and thus nitrogenase) through coupling to the dissipation of some 'energized state' or *protonmotive force* of respiratory (c.q. photosynthetic) energy transducing membranes. As has been mentioned above, the latter workers have convincingly shown that dissipation of the protonmotive force by *artificial* means leads to a shut-down of the electron flow to nitrogenase in all organisms tested: the obligate aerobes *A.vinelandii* and *R.leguminosarum* and the photosynthetic bacterium *Rps.sphaeroides*. Attractive but rather speculative molecular mechanisms have been put forward by Haaker (47) and subsequently

Laane (75). Other mechanisms are however, throughout possible (46); also one should not neglect the hypothesis of Davis and Kotake (26) which postulates that energizable membranes may regulate the Mg^{2+}-concentration in the cytoplasm. This would be especially relevant in further evaluations of the hypothesis of Haaker *et al.* (47) and Laane (75); though these workers have carefully checked that during their manipulations of the membrane protonmotive force the ratio ATP/ADP in the cytoplasm remained virtually constant, in fact it is the ratio MgATP/MgADP rather than the ratio ATP/ADP which is important in nitrogenase activity. Since the affinity of Mg^{2+} for ATP is about ten times that for ADP, the ratio MgATP/MgADP at constant ATP/ADP will depend strongly on the concentration of Mg^{2+}. Still, the dramatic effects of some uncouplers and ionophores on nitrogenase activity *in vivo* as observed by Haaker *et al.* (50) and Laane *et al.* (78, 79) can probably not be wholly explained by changes in the concentration of free Mg^{2+}.

Not only qualitatively, also quantitatively the hydrolysis of ATP by nitrogenase during turnover is not understood. It is indicated in the above reaction stoichiometry (Eq. 1) that 2 molecules of ATP are hydrolysed per 1 electron transferred to substrate. This, however, is the barest minimum attainable *in vitro* (34). It is well established that the consumption of ATP depends strongly on conditions: the ratio of Fe protein over FeMo protein (80), the rate of electron transfer (81), temperature (134) and pH (137). Why this should be so is not at all evident; for a discussion see Miller *et al.*

Especially when considering the ATP requirement of nitrogenase and its consequences for cellular metabolism, caution should be exerted. As has been stated above (I.1), a problem inevitably linked with nitrogenase studies is that one can hardly ever be sure that the nitrogenase one has isolated really represents a physiological preparation, owing to the (oxygen)- lability of the enzyme. Studies of Braaksma *et al.* (11,12) have made dramatically clear that much of the published nitrogenase enzymology may relate only to enzyme preparations with low activity. Therefore, any extrapolation from the *in vitro* to the *in vivo* situation can be made only with caution. This is emphasized by observations of Dalton and Postgate (25a) on the economics *in vivo* of *A.chroococcum* where an upper limit of 4-5 moles ATP consumed per mole N_2 fixed was reported (cf. Eq. 1!).

I.2.3 Maintenance of anaerobiosis

In Postgate's perspective (107,108) the recognition by biologists of the oxygen sensitivity of the nitrogenase system has been one of the landmarks in nitrogen fixation research; and together with the introduction of the C_2H_2 reduction assay it has been instrumental in sparking off the present-day boom. Indeed, the first demonstration in 1960 of *reproducible* nitrogen fixation in cell-free extracts of *C.pasteurianum* using pyruvate as a source of both ATP and reducing equivalents (I.2.2) was only possible through that recognition (22). Thus observations by Bach *et al.* (4) almost thirty years before, putatively demonstrating N_2 fixation in cell-free extracts from *A.chroococcum*, almost certainly have been in error and could not be reproduced even then (18). Initially, attempts at purification of the enzyme were even more bothersome since, in the early sixties, adequate anaerobic handling techniques were not easily available. The solution proved simple. Indeed, as Postgate states (108): 'how fortunate..... that the enzyme tolerated sodium dithionite -an oxygen scavenger which still papers over many of the faults in simple nitrogenase purification systems...! *

Yates and Jones (144) in their review quite strongly defend and augment the postulate that nitrogen fixation is essentially an anaerobic process. On the other hand, however, it is well known that many nitrogen fixers grow either obligatory or preferentially with oxygen present. How do such aerobe fixers, - of which *Azotobacter* is the most prominent example - reconcile their apparently contradictory wants? In recent reviews (42,112), several strategies to do this have been outlined. These will be summarily discussed below.

(a) *Physical barriers.* Several free-living aerobic nitrogen fixers surround themselves with a thick capsule of slime. There is no direct proof that this limits the diffusion of oxygen into the cells (20,145). Similarly, most cyanobacterial fixers have their nitrogen fixing apparatus located in special thick-walled cells, called heterocysts (21), but again there is no proof that this would limit the diffusion of oxygen although it has been claimed (63). A very elegant and well-documented case of a physical barrier, however, exists in the *Rhizobium*-legume symbioses. In this case, the 'barrier' (or buffer, rather) consists of an oxygen-binding protein, called *leghaemoglobin* (LHb) which surrounds the bacteroids

*Even so, one should remain hesitant in equating dithionite-reduced nitrogenase preparations to nitrogenase *in vivo*. Certainly dithionite-reduced Fe- and FeMo proteins should not be considered blandly as the 'normal' forms of these proteins, as Postgate rightly cautions (108). Similarly, key enzymological findings that have been obtained using dithionite as a reductant, should be re-checked using the physiological reductants (ferredoxin or flavodoxin, cf. chapter I.2.2) as electron donor.

in ample amounts (7). The properties of this buffer are such that a high flux of oxygen can be maintained at an extremely low concentration of *free* oxygen (7,138). Actually, the existence of LHb allows one a fascinating glimpse of the intricacies of rhizobium-leguminous symbioses. It has been well established that apo-LHb is coded for and synthesized by the protein-synthesizing machinery from the *plant*, not the bacterium, whereas LHb-haem synthesis probably is the exclusive domain of the bacterium (9). Therefore one can disagree about whether this strategy was evolved by the bacterium or by the plant ?! Indeed, the bacterium by itself ('*ex planta*') has long been considered not to be able to fix nitrogen at all; only relatively recently has it been shown that some strains can fix nitrogen, though only at low external oxygen concentration and with rapidly respirable substrates (8,74,87,101).

(b) *'Respiratory protection'*. As hinted above, microaerophilic nitrogen fixers such as *Rhizobium* spp. *ex planta* and a diverse collection of diazotrophs of minor importance (see Robson and Postgate, 112) require O_2 for nitrogen fixation, but at such low concentrations that their own respiration (or other O_2-consuming processes) can keep internal oxygen concentrations virtually zero (8). *Azotobacter* spp. are extreme examples of such a strategy that is intended to remove any external oxygen by respiration. Indeed, *Azotobacter* spp. have respiratory capacities that are high in the microbial world (136); furthermore, they can adapt their respiration rates to a surprisingly large range of external oxygen concentrations: typically, rates may increase fivefold when the pO_2 is increased from 0.05 to 0.55 atm (25). Apparently, oxygen regulates the composition of the respiratory chain, to the effect that at high aeration energy conservation site III may be passed by (55), and the efficiency of energy conservation at site I is lowered (77), thus boosting O_2 consumption. For carbon substrates taken up in an energy-dependent fashion, such as sucrose, the rate of transport may be involved in respiratory protection (52).

Azotobacter spp. may by the only truly aerobic diazotrophs in the sense that they can adapt to a wide range of pO_2's. The above-mentioned microaerophiles appear not to have this capability (112). The versatility of *Azotobacter* spp. in combination with the diverse effects of O_2 and N_2 on growth and energy conversion efficiency, which have given cause for wonder for a long time (18,112,144) now in any case makes teleological sense through the concept of respiratory protection. However, a complete picture of the molecular mechanisms involved cannot yet be given; other systems in the cell beside the respiratory chain will play a role. As Robson and Postgate surmise (112), one should envision an orchestrated series of changes, both genetic and kinetic, rather than one simple mechanism.

Isolation and characterization of mutants affected in oxygen- or nitrogen-metabolism should prove most useful.

Sometimes the apparent obligatory evolution of hydrogen during nitrogenase catalysis (see above) is assigned an unexpected usefulness, as follows: H_2 might be fed into the respiratory chain *via* an uptake hydrogenase, so that (a) along the way ATP might by synthesized (indeed, in *A.vinelandii* a fourth energy conservation site has been proposed at the level of hydrogenase (77)) and (b) finally O_2 would be reduced to water. The latter would of course contribute to removal of O_2 from the cell like any fast respirable substrate would; however, it would appear a rather artificial construction to convert the waste of reducing equivalents inherent to nitrogenase catalysis into a 'useful' functionality, as Gallon (42) does. After all, the same reducing equivalents might have equally well been fed straight away into the respiratory chain without first passing nitrogenase and in the process consuming > 2ATP/e^-. In any case, H_2 evolution and consumption would hardly contribute significantly to respiratory protection in a true aerobic diazotroph such as *A.vinelandii*.

(c) *Conformational protection*. When cultures of *Azotobacter* spp. are shifted *suddenly* from low to high aeration, nitrogen fixation quickly stops; if subsequently, not having given time for adaptation, the cells are again shifted to the original low aeration, nitrogen fixation will equally quickly reappear. This phenomenon has been termed *switch-off/on* (30,31,140). Both switch-off and switch-on may be accomplished within a few minutes; switch-on occurs also in the presence of chloramphenicol (31). Clearly, a fully reversible process with no protein synthesis involved results in a reversibly inactivated nitrogenase, which in that state is highly tolerant towards oxygen*. From the beginning it has been assumed that the switch-off/on phenomenon had some relation to the fact that nitrogenase in *Azotobacter* cell-free extracts was relatively insensitive to oxygen (31) and could even be partially purified without the stringent anaerobic procedures necessary in handling other cell-free extracts (16). However, it was not at all clear how this special oxygen tolerant form or 'conformationally protected' nitrogenase should be envisioned. Early proposals included binding to membranes (96,110), and stabilization by various crude extract fractions such as NADH dehydrogenase (139) or divalent cations (142). The most elegantly simple and effective explanation has been offered by Haaker *et al.* (51) and Haaker and Veeger (53) who started from a partially purified *A.vinelandii* nitrogenase,

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*Oxygen-dependent switch-off and/or switch-on phenomena have also been shown in *Derxia gummosa* and *Xanthobacter flavus* (142a) as well as in *A.cylindrica* (122a) Even in the 'obligate' anaerobe *C.pasteurianum* a similar phenomenon may exist, -of course at appropriately low O_2-concentrations (ref. 108a, pp. 97-98).

the so-called Bulen-LeComte preparation (16). This preparation is still as stable as the cell-free extract; however, analysis by polyacrylamide gel electrophoresis showed that it contained but one major polypeptide band besides the Av_1 and Av_2 polypeptides. This polypeptide was subsequently shown to be identical to an [2Fe-2S] protein that had been characterized years before by Shethna *et al.* (117). Reconstitution experiments showed that the 'Shethna protein' indeed could protect the nitrogenase proteins. In the present thesis, this finding is confirmed and enlarged upon.

(d) *Oxygen regulation of nitrogenase synthesis.* Any free oxygen which cannot be kept out of the cell passively (strategy *a*) or removed metabolically (strategy *b*) will inactivate the nitrogenase proteins, either reversibly (strategy *c*) or irreversibly (which is no strategy at all). Therefore, it is not surprising that oxygen inside the cell will act as an efficient repressor of nitrogenase synthesis. Repression of nitrogenase synthesis by oxygen has now been confirmed for the microaerophilic *R.japonicum* (112), the facultative anaerobe *K.pneumoniae* (35) and the aerophilic *A.chroococcum* (35). Repression may be as fast *(K.pneumoniae)* or faster *(A.chroococcum)* than that by NH_4^+ (35). In *K. pneumoniae* all the *nif*-genes appear repressable by O_2, but the operon for the structural proteins (*nif* HDKY) to a far greater extent than the regulatory operon (*nif* LA) (29). Although in recent genetic studies emphasis tends to be on the mechanism of repression by NH_4^+ (32,100), a consensus seems to have been reached that the *nif* L product is involved in both O_2- and (to a lesser extent) NH_4^+-repression (66,69,88). Like in NH_4^+-repression, the actual agent which mediates oxygen-repression has not been identified yet, -in any case the two primary signals (NH_4^+ and O_2) appear to employ *different* mediators (35). It might be suggested here that the hydroquinone form of flavodoxin - a nif gene product in *K.pneumoniae* (95)- would be a potentially very sensitive mediator of free oxygen in the cell, since it is extremely rapidly autoxidized (86).

(e) *Various strategies.* Especially cyanobacterial diazotrophs have had to design some smart strategies in order to protect their nitrogenase: since these organisms have a plant-type photosynthesis, they evolve necessarily oxygen inside their cells, augmenting any normal environmental O_2 stress. Apart from the above-mentioned strategies which they may or may not have, the cyanobacteria have developed two exclusive strategies which effectively keep photosynthesis apart from nitrogen fixation: (1) spatial separation and (2) temporal separation. *Spatial separation* means that nitrogenase and photosynthesis are locked into their own specialized cells: 'vegetative' cells for photosynthesis, heterocysts (cf. strategy *a*) for nitrogen fixation. *Temporal separation* so far has been found

only in non-heterocystous cyanobacteria. It means that, given a suitable light-dark regime, photosynthesis will be limited to the light period, nitrogen fixatio to the dark. These and other aspects of cyanobacterial nitrogen fixation have been reviewed extensively elsewhere (42,125). It is justifiable that this class of diazotrophs should receive a great deal of attention in the literature since it is both agriculturally and biotechnologically a very promising class (123,41).

The dualistic role which O_2 appears to play in Nature, beneficial as well as detrimental, is a fascinating subject which only recently is coming to flower. Insight in mechanistic aspects, however, is still very sketchy. A review has been compiled recently by Elstner (39); several excellent articles may be found in the volume Strategies of Microbial Life in Extreme Environments' (118). No published work at all exists on the chemistry of the destructive reaction of O_2 with nitrogenase proteins, -which is unfortunate to say the least.

I.2.4 Concluding remarks

Completing, at last, the discussion on 'costs' involved in nitrogen fixation, it should be clear that a lot of work is left to be done yet. Apart from the minimum amount of ATP which is directly required in nitrogenase function (2 ATP/e^-) and indirectly spent in the generation of reducing equivalents (3 ATP/e^-, assuming 3 energy conservation sites and complete coupling), an unknown amount of energy may be spent in : (1) the 'pushing' of electrons from NAD(P)H to the primary electron donor for nitrogenase (cf. I.2.2), and (2) protection of the nitrogenase proteins against inactivation by surplus oxygen (I.2.3). Last but not least, the cost involved in *de novo* protein synthesis may be considerable. It has been calculated that the nitrogenase structural proteins alone may make up as much as 10% of the content of soluble proteins in a diazotroph (35,111). Since the *nif* gene cluster contains *at least* 12 genes apart from those coding for the structural proteins (69), and perhaps many more are involved in nitrogen fixation by symbiotic diazotrophs, this last component of the costs of nitrogen fixation may indeed be high.

To arrive at a more satisfactory and conclusive picture of how nitrogenase function fits into cellular metabolism and what costs are involved, however, more precise data are needed, from both *in vitro* and *in vivo* studies. For more detailed discussions of the presently available data, see Gutschick (45), Pate *et al.* (104), Postgate *et al.* (109) and Schubert (115).

I.3. Outline of this thesis

Following the pioneering physiological studies of Haaker *et al.* (47,53) on the generation of reducing equivalents for nitrogenase in *Azotobacter vinelandii*, it seemed desirable to further characterize interactions between the likely ultimate electron donors (flavodoxin and/or ferredoxin, see also ref. 145a) and nitrogenase. Isolation of these proteins was therefore necessary. Flavo- and ferredoxin from *A.vinelandii* have been prepared to purity; as far as nitrogenase was concerned, however, it was deemed that the O_2-tolerant nitrogenase complex as isolated originally by Bulen and LeComte (16) should render information more physiologically relevant than could be the case using highly purified nitrogenase components (Av_1 + Av_2). In chapter II, results are described suggesting that the Bulen-LeComte nitrogenase complex, with fully reduced flavodoxin as a source of reducing equivalents, has regulatory properties not exhibited by a more highly purified (Av_1 + Av_2) complex, due to the presence of a 'contaminating' third protein.

This third protein appeared to be the same as that shown by Haaker *et al.* (51, 53) to be responsible for the oxygen-tolerance of the nitrogenase complex.

The spectral properties and molecular weight are shown in chapter II to be identical to that of the [2Fe-2S] ferredoxin isolated earlier by Shethna *et al.* (117) who, however, did not ascribe any physiological function to this protein.

In chapters III and IV, the interactions between Av_1, Av_2 and the third protein are further characterized, as well as the conditions necessary to induce the reconstitution of an oxygen-tolerant complex from the purified proteins. The relation between the oxygen-tolerant complex and the so-called 'switched-off state' of nitrogenase activity *in vivo* is discussed.

Please note. Chapter III is the result of a multi-author effort. The author of the present thesis takes responsibility for the 2nd part only, 'Protection of *A.vinelandii* N_2 ase against damage by O_2'.

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II. REGULATION OF NITROGEN FIXATION BY Fe-S PROTEIN II IN *AZOTOBACTER VINELANDII*

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1. Several low-potential electron carriers from different sources can be photoreduced by the system 3,10-dimethyl-5-deazaisoalloxazine/*N*-tris(hydroxymethyl)methylglycine. The carriers studied were flavodoxin, ferredoxin I and iron-sulfur protein II from *Azotobacter vinelandii* and the flavodoxins from *Desulfovibrio vulgaris* and *Peptostreptococcus elsdenii*.

2. Electron transport to *A. vinelandii* nitrogenase was studied, employing different preparations of the enzyme: a crude complex; a complex reconstituted from the 0.27 M and 0.38 M NaCl fractions after DEAE-cellulose chromatography; a complex reconstituted from the 0.27 M and 0.38 M NaCl fraction plus iron-sulfur protein II purified from the 0.15 M NaCl fraction. Of all photoreduced carriers tested, only flavodoxin hydroquinone from *A. vinelandii* catalyzes significant electron transport to these complexes.

3. The time course of oxidation of substrate-amounts of *A. vinelandii* flavodoxin hydroquinone by catalytic amounts of crude nitrogenase complex shows three characteristic phases: an initial lag phase (1), a phase with constant rate over a range of redox potentials (2) and a final phase with rapidly declining rate (3). It was shown that the Fe-S protein II is responsible for the lag phase; the potential where phase 2 changes into phase 3 is at a higher value in the presence of Fe-S protein II. Pre-reduction of the enzyme photochemically abolishes phase 1 and causes phase 2 to proceed at a higher rate. The rate in phase 2 can be enhanced also by lowering the 'starting potential' of the flavodoxin hydroquinone/semiquinone couple.

4. *A. vinelandii* flavodoxin shuttles between its hydroquinone and semiquinone forms during steady-state electron transfer. Over 90% of the reducing equivalents is recovered in ethylene formed from acetylene. The concentrations of flavodoxin hydroquinone to give half-maximum rate in the acetylene reduction assay is 3–6 μM. A scheme is proposed for the regulation of electron donation to nitrogenase.

It is well established that pyruvate is a main source of reducing equivalents for nitrogenase in many aerobic nitrogen-fixing bacteria, and that electrons are usually transferred from pyruvate dehydrogenase *via* ferredoxin [1]. Benemann *et al.* [2] as well as Yoch and Arnon [3] considered ferredoxin a possible candidate as electron donor for nitrogen fixation in *Azotobacter*, but attempts to demonstrate pyruvate-ferredoxin oxidoreductase activity in extracts have given equivocal results [4].

The role of flavodoxin in aerobic nitrogen-fixing organisms is also not certain. As in aerobic organisms it might substitute for ferredoxin under conditions of iron-deficiency. Benemann *et al.* [2,5] were the first to postulate a role of flavodoxin in nitrogen fixation of *Azotobacter*. They showed that flavodoxin could couple the reducing power of chloroplast photosystem I to nitrogenase in a cell-free extract. However, the activity obtained amounted to only a fraction of the activity with dithionite as electron donor. In addition, the specificity of the reaction seemed low, *e.g.* flavodoxin from *Anacystis nidulans* (phytoflavin) was shown to be more effective in this reaction than *Azotobacter* flavodoxin [6]. Yates provided more direct evidence by using purified nitrogenase components and dithionite-reduced *Azotobacter* flavodoxin [7]. The reduced flavodoxin was reoxidized by *Azotobacter* nitrogenase, in the presence of ATP, to the semiquinone, and the reaction occurred at a reasonable rate. This result suggested that *Azotobacter* flavodoxin transmits electrons directly to the nitrogenase without the need for intermediate carriers. However, the presence of dithionite in these experiments makes the results

Trivial Names. Tricine, *N*-tris(hydroxymethyl)methylglycine; deazaflavin; 3, 10-dimethyl-5-deazaisoalloxazine.

questionable. Mayhew in our laboratory has shown recently [8] that a redox equilibrium exists between reduced flavodoxin and the oxidation product(s) of dithionite, presumably as follows:

$$FH_2 + HSO_3^- \rightleftharpoons FH^\cdot + SO_2^{\cdot-} + H_2O \quad (pH < 7). \tag{1}$$

$$FH^- + HSO_3^- \rightleftharpoons FH^\cdot + SO_2^{\cdot-} + OH^- \quad (pH < 7). \tag{2}$$

It is thus clear that $SO_2^{\cdot-}$ may have served as an intermediate carrier in Yates' experiments.

Therefore we studied the electron transport between several low-potential electron carriers and *Azotobacter* nitrogenase without using dithionite. Photoreduction with EDTA is a well-known alternative to chemical reduction for flavoproteins, but it does not lead to full reduction of *Azotobacter* flavodoxin [9]. We used a 5-deazaflavin which appears to be a superior photoreductant in the presence of a nitrogen-containing donor, such as EDTA or tricine (V. Massey, personal communication).

MATERIALS AND METHODS

Growth Conditions and Enzyme Preparations

Azotobacter vinelandii ATCC 478 was grown and harvested as described by Bresters *et al.* [10]. Flavodoxin and ferredoxin I from this organism were purified according to Yoch and Arnon [3].

A. vinelandii nitrogenase complex was purified according to Bulen and Le Comte [11] up to the second $MgCl_2$ precipitation step. This so-called C_{42}-I preparation had a specific activity of 100 – 180 nmol acetylene reduced min^{-1} mg^{-1} with dithionite as electron donor. When the components needed to be separated, preparation C_{42}-I was chromatographed on DEAE-32 cellulose [11] in the presence of 2 mM dithionite. Dithionite-free crude *A. vinelandii* component 1 and component 2 were obtained by anaerobic gel filtration on Sephadex G-25, medium grade. Specific activities of crude components separately have not been measured but the reconstituted mixtures used in some experiments reported here, with a crude component 1/component crude 2 = 4/1 (w/w) ratio, had specific activities (based on total protein) of 160 nmol C_2H_4 $mg^{-1} \cdot min^{-1}$. The gel electrophoresis patterns of the preparations will be published elsewhere [12].

Iron-sulfur protein II [13] was purified from preparation C_{42}-I as described in Results and Discussion. Reconstitution of the nitrogenase components with iron-sulfur protein II was performed as described by Haaker and Veeger [14] under optimum conditions and by incubation during 30 min.

Flavodoxins from *Desulfovibrio vulgaris* and *Peptostreptococcus elsdenii* were kindly donated by Dr S. G. Mayhew.

Heated chloroplast fragments were prepared from climate-room-grown spinach as described by Yoch and Arnon [15].

Analytical Methods

Standard acetylene assays were run at 30 °C in 6.5-ml bottles sealed with a Subaseal cap, and contained a mixture of 25 mM tricine buffer, 1 mM ATP, 2 mM $MgCl_2$, 10 mM creatine-phosphate and 5 U creatine kinase (creatine and ATP as substrates), final pH 7.5. This mixture was flushed for at least 30 min with argon, which had been purified by passage over a heated BASF catalyst. Acetylene (10%) and 20 mM dithionite were then added. The mixture was equilibrated for 10 min with the gas phase to remove the last traces of oxygen, after which the nitrogenase preparation was added and the ethylene produced was measured as described earlier [16]. Several amounts of a nitrogenase preparation were analysed to exclude an underestimation of the nitrogenase activity caused by low nitrogenase concentrations (dilution effect).

Photoreduction of electron carriers was carried out in the presence of 50 mM tricine buffer, 50 mM 2[N-morpholino]ethanesulfonic acid buffer, 2 mM dithiothreitol, 2 mM $MgCl_2$, 20 U/ml glucose oxidase, 20 mM glucose, 1000 U/ml catalase, 5 μM 3,10-dimethyl-5-deazaisoalloxazine (deazaflavin) and carrier at the concentrations indicated, final pH 7.3. This mixture was flushed with argon for 20 min, shaken for 30 min in the dark and then irradiated with white light for 10 min. Light intensity was 15 mW/cm^2 measured between 400 – 500 nm by a plane solarimeter.

Nitrogenase activity was measured by three different methods.

Method 1. Electron transfer from substrate-amounts of photoreduced carriers *via* nitrogenase to H^+. In this case the photoreduction mixture was supplemented with 10 mM creatine phosphate and 10 U/ml creatine kinase (creatine and ATP as substrates). The reaction was started by injecting appropriate amounts of nitrogenase into the reaction vessel and followed by measuring the increase in absorbancy at room temperature. Activity *versus* redox potential plots in the case of *A. vinelandii* flavodoxin were calculated from representative time courses. At different times during a single assay redoxpotentials were calculated from the semiquinone/hydroquinone ratio, based on $\Delta A_{580\,nm}$ *A. vinelandii* flavodoxin 5400 M^{-1} cm^{-1} [17]. Rates of hydroquinone oxidation were determined from tangents to the trace and recalculated into a percentage of dithionite activity, as measured

under standard assay conditions, by dividing them by 2.

Method 2. Stoichiometry of donor oxidation and acetylene reduction. This was determined with 20% acetylene present. Before the reaction was started the mixture was thoroughly equilibrated with C_2H_2. After the reaction again the mixture was thoroughly shaken and the amount of C_2H_4 formed determined by gas chromatography.

Method 3. Reduction of acetylene in the presence of catalytic amounts of electron carriers, deazaflavin and continuous illumination. These experiments were performed in thin-walled 22.5-ml glass bottles at 30 °C, containing the mixture described above but with 80 μM deazaflavin and 20% acetylene. This concentration of deazaflavin was saturating under the conditions used. Anaerobic conditions were obtained as described above. Before starting the reaction by injecting 10 μl of preparation C_{42}-I, the mixture was pre-illuminated for 10 min to remove the last traces of O_2. The reaction was either followed with time by analyzing gas samples during the reaction, or stopped after 10 min by $HClO_4$, whereafter the total amount of the ethylene formed was determined. The two methods gave identical results indicating that equilibrium between liquid and gas phase was established fast enough. When instead of deazaflavin, chloroplast fragments were used, the assay mixture described in [15] was used, supplemented by 20 U/ml glucose oxidase, 20 mM glucose, and 1000 U/ml catalase. All reactions were carried out under argon which had passed heated BASF catalyst. Acetylene was purified by storage for at least 24 h in the presence of Fieser solution.

Protein concentrations were determined by the biuret method. Optical spectra were recorded in a Cary model 1605, circular dichroism spectra in a Roussel-Jouan dichrograph and electron paramagnetic resonance spectra in a Varian model E-3.

Biochemicals

All enzymes and cofactors were purchased from Boehringer. 5-Deazaflavin was a gift from Dr V. Massey and Dr P. Hemmerich, and synthesized as published recently [18].

RESULTS AND DISCUSSION

Electron Transfer between Carriers and Nitrogenase

Several reports have appeared in the literature during the last 7 years on attempts to assess the effectiveness of different low-potential electron carriers as donors for nitrogenase by linking them to the reducing power of chloroplasts (*e.g.* [6,19–21]). However, in our opinion this method cannot give a reliable indication of the actual functioning *in vivo* of a certain carrier as the ultimate donor for nitrogenase, because firstly, if activity is observed, one has to consider that chloroplast fragments are able to generate a redox potential of at least −700 mV [22], a value which can hardly be expected in heterotrophic cells; and because secondly, if activity is not observed, low activity with either chloroplasts or nitrogenase may be the cause of bad functioning. Photochemical reduction with an artificial system permits an assessment of the second objection, since the reaction mixture is optically clear and consequently reduction and oxidation of any component can be easily followed spectroscopically.

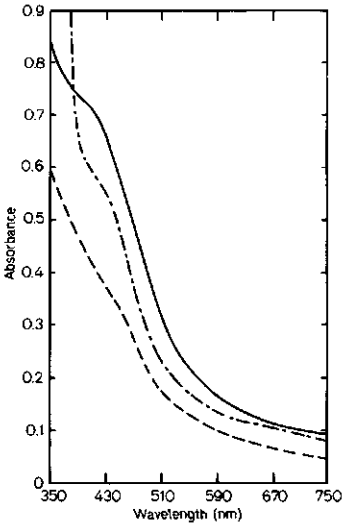

Fig. 1. *Spectrum of oxidized and photoreduced* A. vinelandii *ferredoxin I compared with that of dithionite-reduced ferredoxin I.* The conditions for photoreduction are described in Methods; reduction by 10 mM $Na_2S_2O_4$ was done anaerobically in the same mixture. (——) oxidized carrier; (– – – –) after photoreduction during 10 min; (–·–·–) after reduction by $Na_2S_2O_4$ for 80 min

Flavodoxins from *A. vinelandii*, *Peptostreptococcus elsdenii* and *Desulfovibrio vulgaris*, ferredoxin, ferredoxin I and iron-sulfur protein II from *A. vinelandii* as well as methylviologen can be reduced rapidly and reversibly by photoreduction with mixtures of deazaflavin and tricine. The spectra of the photoreduced carriers are identical with those already published in the literature, except that of ferredoxin I (Fig. 1). The spectrum of flavodoxin hydroquinone has been registered only at alkaline pH in the presence of a large excess of $Na_2S_2O_4$ (*cf.* [9]). Ferredoxin I can be bleached this way to a far greater extent in the 400-nm region than with $Na_2S_2O_4$ (*cf.* [19]); the reduction is rever-

sible. This casts some doubt on the previously published value of the E'_m [3]. After one night dialysis against 10 mM tricine/KOH, pH 7.2, the absorption at 400 nm was recovered by 84% after photoreduction and 77% after dithionite reduction.

Table 1 shows a comparison between the two methods of photoreduction with several electron carriers. These data show that, although ferredoxin I is the best electron donor for nitrogenase with the chloroplast system, in fact a low concentration (10 μM) of flavodoxin is far superior in the system with deazaflavin.

Table 1. *Comparison of the efficiency of several electron donors in the acetylene reduction by preparation C_{42}-I, employing two different photoreductants*

Data are expressed as percentage acetylene reduction rate with dithionite as electron donor. In the standard assay this activity was 180 nmol mg^{-1} min^{-1}. Donors were added to a final concentration of 10 μM and purified from *A. vinelandii* except where indicated. Other conditions as described in Methods. n.d. = not determined

Donor(s)	Photoreductant	
	chloroplasts/ 2,6 dichloro-indophenol/ ascorbate	deazaflavin/ tricine
	%	
Flavodoxin	0	80
P. elsdenii flavodoxin	7	16
D. vulgaris flavodoxin	n.d.	15
Flavodoxin + *P. elsdenii* flavodoxin	16	111
Ferredoxin I	33	13
Iron-sulfur protein II	11	6
Flavodoxin + ferredoxin I	56	89
None	0	3

Experiments with substrate-amounts of photoreduced carriers and catalytic amounts of nitrogenase support this evidence. In such experiments the redox potential of the donor system is a quantity that can be easily calculated from the known midpoint potentials, thereby overcoming the first objection mentioned above. Fig. 2 shows the kinetics of such a reaction of nitrogenase with different types of photochemically-reduced donors (*e.g.* [23]). Midpoint potentials of the donors used have been reported in the literature (*A. vinelandii* flavodoxin − 495 mV at pH 8.2 [17], *D. vulgaris* flavodoxin − 440 mV at pH 7.8 [17, 24], *P. elsdenii* flavodoxin − 370 mV at pH 7 [25], methylviologen − 460 mV [26]). It is obvious (Fig. 2) that only flavodoxin hydroquinone of *A. vinelandii* is oxidized rapidly by nitrogenase to a high semiquinone/hydroquinone ratio, while the flavodoxin hydroquinones from *D. vulgaris* and *P. elsdenii* are oxidized slowly and only to a small extent, even in the presence of a larger nitrogenase concentration. The slow secondary oxidation as is apparent in Fig. 2B and C after the initial jump, is not related to nitrogenase turnover and is probably caused by traces of oxygen. The coupling between *A. vinelandii* flavodoxin hydroquinone oxidation and acetylene reduction is high; usually more than 90% of the reducing equivalents are recovered in ethylene. Methylviologen radical is also oxidized to a high extent by nitrogenase, but much higher enzyme concentrations are necessary to obtain measurable rates. This is not unreasonable since nitrogenase at low concentrations exhibits a so-called dilution effect (see Methods). The high rates with

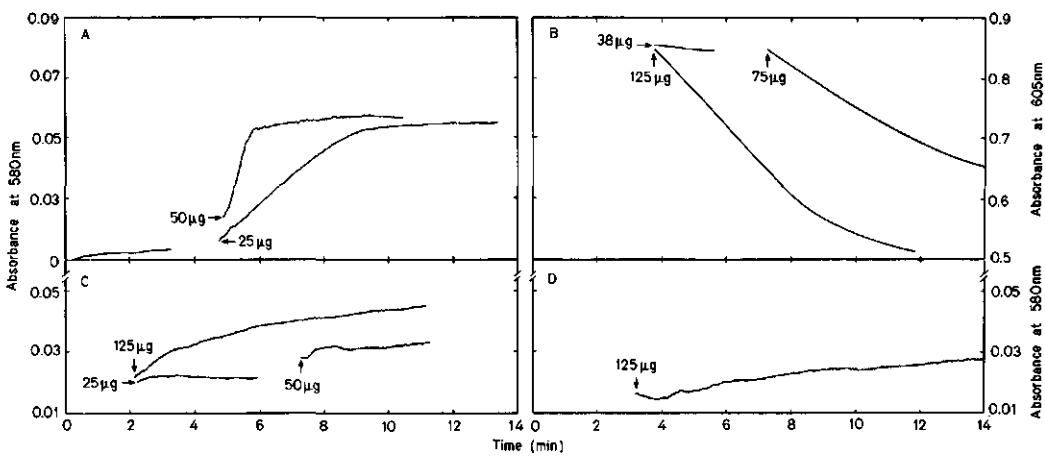

Fig. 2. *Time course of enzymatic and non-enzymatic oxidation of photoreduced electron carriers.* Flavodoxins were from the following organisms: (A) *A. vinelandii* (0.3 mg/ml); (C) *P. elsdenii* (0.2 mg/ml); (D) *D. vulgaris* (0.2 mg/ml); (B) represents the oxidation of methylviologen semiquinone (0.2 mM). Different amounts of preparation C_{42}-I (12.5 mg/ml) were added at the times indicated by the arrows. Photoreduction was performed in the presence of 10 μM deazaflavin as described under Methods

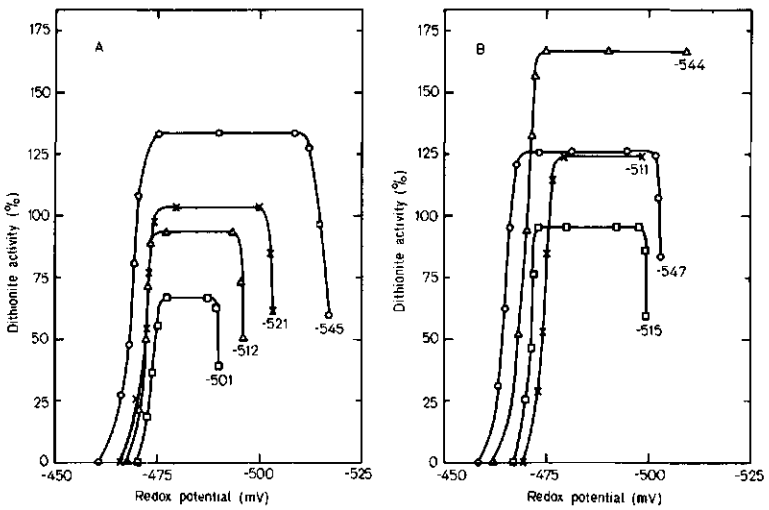

Fig. 3. *Activity-redox potential relation of flavodoxin hydroquinone oxidation by nitrogenase under different conditions.* 60 nmol of *A. vinelandii* flavodoxin in a total volume of 0.6 ml were photoreduced and reoxidized to different extents ('starting potentials') by injecting air-saturated water. The reaction was started by addition of 85 µg preparation C_{42}-I. Curves were drawn according to the procedure described in Methods. The starting potentials for each experiment are given in the figure in mV. (A) Without pre-treatment; (B) treatment of preparation C_{42}-I: (O——O, □——□) no treatment; (Δ——Δ, ×——×) irradiation for 2–3 min in the presence of 5 µM deazaflavin and 10 µg/ml *A. vinelandii* flavodoxin. The point of zero activity is not critically dependent on the correct absorption coefficient at 580 nm of the flavodoxin semiquinone. These curves are based on $\varepsilon_{580} = 5400\ M^{-1}\ cm^{-1}$, but if $5200\ M^{-1}\ cm^{-1}$ is assumed (Fig. 7 of [23]) the point of zero activity shifts by only + 4 mV

Azotobacter flavodoxin using low concentrations of nitrogenase are consistent with the finding of Yates [27] that *A. chroococcum* nitrogenase did not show a dilution effect with flavodoxin present. Photoreduced *A. vinelandii* ferredoxin I is not oxidized measurably by nitrogenase (not shown) in accordance with Yates' finding [7] with $Na_2S_2O_4$-reduced ferredoxin I; also photoreduced iron-sulfur protein II is not oxidized by nitrogenase.

By varying the concentration of flavodoxin hydroquinone in the acetylene reduction assay performed under constant illumination (80 µM deazaflavin) we were able to estimate that 50% of the maximum velocity occurs at a flavodoxin hydroquinone concentration of 3–6 µM. The maximum rate calculated for infinite *A. vinelandii* flavodoxin hydroquinone concentration was 150% of the activity observed under similar conditions with $Na_2S_2O_4$. For the flavodoxin of *P. elsdenii* and *D. vulgaris* these rates are 30% and 15% respectively of the activity with the *A. vinelandii* carrier.

The question arises whether 'fit' or redox potential determines the differences in reactivity of different electron carriers in the systems used in Table 1 and Fig. 2. With *A. vinelandii* flavodoxin we can calculate (see below) the lowest redox potential at which the rate of electron transfer becomes zero; this is − 460 to − 470 mV (*cf.* Fig. 3). If the same is true for other donors, it is easily understood why in experiments with substrate-amounts of electron carrier such as those of Fig. 2B and C little or no electron transfer is observed. The same is true for other carriers. Accepting the values of the midpoint redox potentials published for the different carriers, the − 460 mV limit is exceeded at ratios of oxidized to reduced carriers of 0.003 for *P. elsdenii* flavodoxin, 0.54 for *D. vulgaris* flavodoxin, 0.22 for *A. vinelandii* ferredoxin I, 4.0 for *A. vinelandii* flavodoxin and 1.0 for methylviologen. Methylviologen apparently reacts differently (Fig. 2B); this carrier is oxidized by nitrogenase to a ratio of about 8. We have no explanation for this observation, but it is in agreement with results obtained for the reaction of *Chromatium* nitrogenase with mediator dyes at fixed redox potentials [28].

We thus conclude that the midpoint redox potential of a given physiological carrier largely determines its reactivity with nitrogenase. An effect of reactivity with nitrogenase seems to be less important but is definitely present. For instance, *A. vinelandii* flavodoxin performs very poorly as electron carrier between chloroplasts and nitrogenase (Table 1), but when *P. elsdenii* flavodoxin is added, the resulting nitrogenase activity is much more than additive. In other words, a redox chain

chloroplasts → *P. elsdenii* flavodoxin → *A. vinelandii* flavodoxin → nitrogenase

is more effective than the same chain without *A. vinelandii* flavodoxin. This suggests that *A. vinelandii* flavodoxin fits the electron input site of nitrogenase better than does *P. elsdenii* flavodoxin. With other carriers (*A. vinelandii* flavodoxin and ferredoxin I) such effects, though less clear, are also observed. The much lower maximum activities observed with the flavodoxins from *P. elsdenii* and *D. vulgaris* under constant illumination (Table 1) emphasize that the 'fit' to the nitrogenase plays an additional role.

Mechanism of Electron Transfer

With regard to the mechanism of electron transfer between flavodoxin and nitrogenase both one-electron and two-electron steps have to be considered. Both lead eventually to formation of flavodoxin semiquinone. However, the latter mechanism requires a non-rate-limiting comproportionation step of hydroquinone and quinone to semiquinone following the nitrogenase-catalyzed reoxidation of hydroquinone to flavin-quinone. The comproportionation reaction should in that case be much faster than the rate of quinone formation, since the spectrum during the oxidation of the hydroquinone does not show evidence for the presence of flavin quinone. We determined the rate of comproportionation by anaerobically mixing photo-reduced *Azotobacter* flavodoxin with different amounts of flavodoxin quinone and following the rate of the reaction at 580 nm. From several experiments a second-order rate constant $k = 8.0 \pm 3.0 \times 10^2\ M^{-1} \cdot s^{-1}$ was calculated. Since an initial overall rate of formation of flavodoxin semiquinone during oxidation of hydroquinone by nitrogenase of $10^{-6}\ M^{(-1)} \cdot s^{-1}$ is easily obtained, one can calculate that the product [hydroquinone] × [quinone] $\approx 10^{-9}\ M^2$ in order to have comproportionation accounting for the reaction rate observed. Taking into account that the concentration of hydroquinone in our experiments was less than 100 μM, the concentration of flavodoxin quinone must be higher than 10 μM. Such a concentration can be easily detected, but was never found. In fact even at the endpoint of the nitrogenase reaction no significant concentration of quinone is present. Thus it can be concluded that a mechanism in which two electrons are transferred simultaneously does not operate in the reduction of nitrogenase by flavodoxin hydroquinone, leaving a one-electron transfer as the actual mechanism.

Regulation of Flavodoxin Hydroquinone Oxidation

The kinetic pattern in Fig. 2A is intriguing in view of the linearity over a wide range of redox potentials of the donor system. In Fig. 3A this is shown more clearly by plotting the rate of hydroquinone oxidation against the redox potential of the donor. The redox potential was calculated from the ratio of semiquinone to hydroquinone, the functional couple in the nitrogenase reaction.

All curves in Fig. 3A show the same pattern, which can be divided into three phases: after an initial lag phase, a second phase with constant reaction rate is observed, which is followed by a phase where the rate rapidly declines. In all cases the reaction rate is zero when the redox potential exceeds − 460 mV. When the starting redox potential of the donor system is adjusted by adding oxygen before starting the reaction, to accomplish partial reoxidation of the highly auto-oxidizable flavodoxin hydroquinone, a dependency on this potential of the rate of oxidation during the second linear phase is observed. In Fig. 3A some rate curves obtained at different starting potentials are given. Plotting the oxidation rate in the second phase against the starting potential yields a curve that indicates a 'working' midpoint potential for *Azotobacter* nitrogenase of − 485 to − 495 mV (not shown). This is somewhat lower than values published previously for redox-equilibrated systems for nitrogenase from *Chromatium* [28] and *Azotobacter* [29]: (− 460 mV).

The fact that the level of the second phase is determined by the starting redox potential, suggests that the nitrogenase preparation (in this case preparation C_{42}-I) is activated by low-potential electrons in order to reach maximum activity. The results of Fig. 3B support this idea. Preparation C_{42}-I was illuminated in the presence of deazaflavin and flavodoxin for about 2 − 3 min, before adding it to the reaction vessel in which the starting redox potential was adjusted as described above. Two effects of pre-reduction are observed: firstly, the reaction rate in the second phase is considerably enhanced, and secondly, the lag phase has disappeared.

Purification of Iron-Sulfur Protein II from Preparation C_{42}-I and Its Regulating Effect on Nitrogenase

In 1966 Bulen and Le Comte [30] found in their purified nitrogenase preparations (C_{42}-I) a pinkish protein fraction, that could be separated from the nitrogenase proteins by DEAE-cellulose chromatography. The fraction could be eluted from the column with 0.15 M NaCl. The authors identified this fraction as cytochromes $c_4 + c_5$. Kelly *et al.* in 1967 [31] confirmed this and reported furthermore a stabilizing and even reactivating effect of this 'cytochrome' fraction on a reconstituted inactive complex stored at 12 °C. Bulen and Le Comte [11] mentioned a tentative identification of this fraction as the pink iron-sulfur protein II, that had been purified from a butanol extract of whole *A. vinelandii* cells by Shethna *et al.*

[13] and for which no physiological function has been found. Haaker and Veeger [14] recently showed that this crude pink fraction from preparation C_{42}-I is responsible for the relatively high stability towards oxygen of preparation C_{42}-I and probably in general in crude extracts of *A. vinelandii*. The pink fraction contained at least four different proteins. One of those was not present in the two other eluates [14]. Formerly this stabilising effect had been ascribed to cytoplasmic membranes [32] or a so-called conformational protection [33]. These stabilizing effects suggested to us that the pink fraction might form a complex with the nitrogenase proteins or otherwise affect catalytic or regulatory sites, thereby inducing the typical pattern of flavodoxin hydroquinone oxidation as described in Fig. 3. We decided therefore to purify the component responsible, to identify it and to test its influence on the nitrogenase.

The 0.15 M NaCl eluate (*cf.* [11]) is concentrated in an Amicon ultrafiltration cell, using a UM 10 filter, and 40 mg brought on a DEAE-cellulose column (11 × 1 cm) equilibrated with water. In contrast to the Bulen-Le Comte complex the colored protein from the 0.15 M NaCl eluate binds very weakly to DEAE-cellulose. The column is washed with 5 mM potassium phosphate buffer, pH 7.4, until the pink color arrives at the bottom of the column, and then it is eluted with 20 mM potassium phosphate buffer, pH 7.4.

Fractions with an A_{280}/A_{344} ratio of less that 1.1 are pooled and concentrated as before. Next 2 ml of a solution with $A_{344} = 2.5$ is brought on a Sephadex G-100 column (36 × 1.3 cm), equilibrated with 20 mM potassium phosphate buffer containing 100 mM KCl, pH 7.4, and eluted with the same buffer. Fractions with an A_{280}/A_{344} ratio of 0.82 are pooled and concentrated. The major protein at this point is more than 90% pure as judged by dodecyl sulfate/gel electrophoresis and the A_{280}/A_{344} ratio of 0.82 (*cf.* [34]). The optical, circular dichroic and electron paramagnetic spectra are shown in Fig. 4. Analytical Sephadex gel filtration gives a molecular weight identical to that of chymotrypsin (molecular weight 24000). According to these data the major protein is identical with the iron-sulfur protein II of Shethna *et al.* [13]. We should remark here that two different sets of electron paramagnetic resonance g-values have been reported in the literature for iron-sulfur protein II [34, 35]. Our values confirm those of DerVartanian *et al.* [34]: $g_x = 1.89$, $g_y = 1.94$, $g_z = 2.035$.

The amount of iron-sulfur protein II in C_{42}-I preparations can be estimated by comparing the intensities of the Cotton effects in circular dichroism spectra of preparation C_{42}-I and pure iron-sulfur protein II. The method is very accurate, since the nitrogenase proteins do not exhibit significant Cotton effects in the visible region [36]; the circular dichroism spectrum of preparation C_{42}-I is thus almost identical

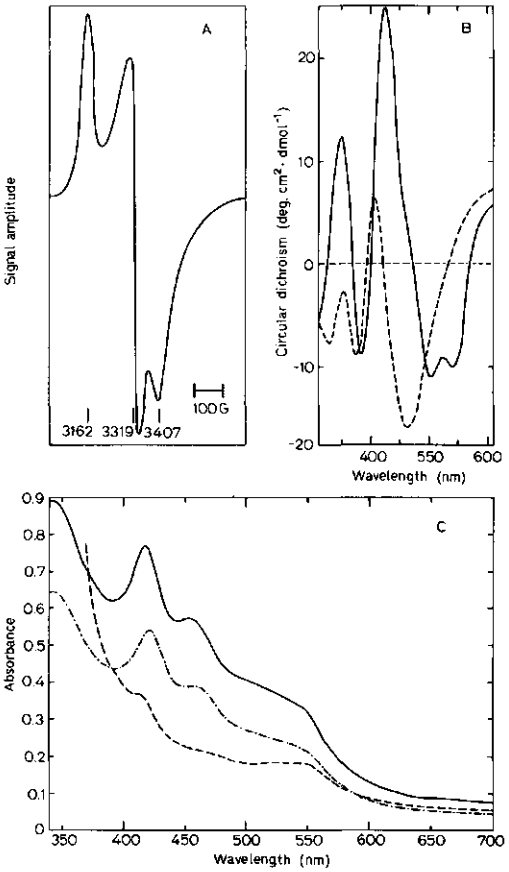

Fig. 4. *Absorption, circular dichroism and electron paramagnetic resonance spectra of the protein fraction purified from preparation C_{42}-I in its oxidized and reduced states.* (A) Electron paramagnetic resonance spectrum of the dithionite-reduced form (4.2 mg · ml^{-1}), (B) circular dichroism spectra and (C) absorption spectra (2.6 mg · ml^{-1}). Symbols in B and C are (——) oxidized form; (– – –) reduced form; (— · —) after 1 h aerobic dialysis of the reduced form. Reduction was done either by addition of 4 mM dithionite or (in the case of optical and circular dichroism spectra) by photoreduction. Photoreduction or dithionite-reduction give indentical spectra, at least outside the range of dithionite absorption. Electron paramagnetic resonance spectra were recorded at liquid nitrogen temperature. Conditions were as follows: microwave power 10 mW, modulation amplitude 10 G, scanning rate 250 G/min, time constant 1 s, microwave frequency 9006 GHz. The field markers are in gauss

with that of iron-sulfur protein II. The results indicate that the iron-sulfur protein II is present in preparation C_{42}-I in a molar ratio of roughly 1:1 to nitrogenase. This value is estimated on the basis that 70% of the weight of preparation C_{42}-I consists of nitrogenase proteins as gel electrophoresis indicates (*cf.* [12, 14]), and using molecular weights of 245000 for component

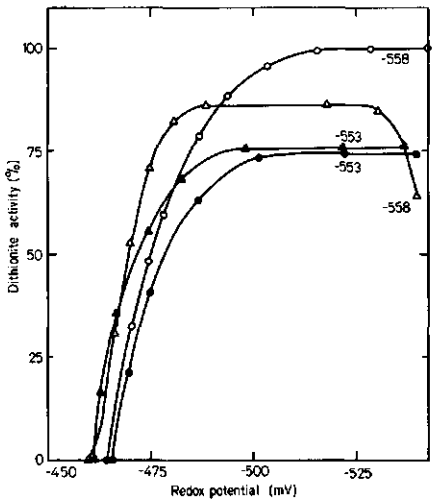

Fig. 5. *Activity-redox potential relation of flavodoxin hydroquinone oxidation by nitrogenase under different conditions.* 60 nmol of *A. vinelandii* flavodoxin in a total volume of 0.6 ml were photoreduced. The reaction was started by addition of enzyme. 100 µg was added of the following preparations: (○——○) a mixture of crude components 1 and 2 (see Methods); (△——△) crude components 1 and 2 plus purified iron-sulfur protein II; (●——●) crude component 1 and 2 that had been irradiated for 5 min in the presence of 5 µM deazaflavin; (▲——▲) crude components 1 and 2 plus purified iron-sulfur protein II that had been irradiated for 5 min in the presence of 5 µM deazaflavin

I [37], 60000 for component II [37] and 24000 for Fe-S protein II [34]. Furthermore, in our experience, about 800 mg of preparation C_{42}-I can be obtained from 1 kg of wet *A. vinelandii* cells, and this amount of preparation C_{42}-I should contain about 50 mg iron-sulfur protein II, a value in excellent agreement with the value of DerVartanian *et al.* [34].

This simple method of purification is an attractive alternative for the procedure described by DerVartanian *et al.* [34], in which the first purification step after extracting the cells with butanol is DEAE-cellulose chromatography, which however binds free iron-sulfur protein II only very weakly. Moreover in our procedure no potentially harmful butanol extraction is needed.

Evidence that the iron-sulfur protein II does regulate nitrogenase activity is shown in Fig. 5. When nitrogenase activity is reconstituted from crude components 1 and 2 (see Methods) no lag phase in the flavodoxin hydroquinone oxidation can be detected; in addition the second phase is severely affected. The reaction rate is much more strongly dependent on the redox potential of the flavodoxin semiquinone/hydroquinone couple. The activity begins to decline even below − 500 mV (*cf.* Fig. 3). Addition of pure iron-sulfur protein II restores the typical pattern that is exhibited by preparation C_{42}-I (*cf.* Fig. 3A). It is also remarkable that the activity of the flavodoxin system is equal to the activity in the presence of $Na_2S_2O_4$ while the activity with preparation C_{42}-I is under optimum conditions about 50% higher (Fig. 3A, B). The lag is present when the system is not pre-reduced. After pre-reduction the activity is slightly lower by comparison with the activity of untreated nitrogenase with or without Fe-S protein II. Pre-reduction does not enhance the rate in the second phase but it abolishes the lag phase as in preparation C_{42}-I (*cf.* Fig. 3B). The lack of enhancement might be ascribed to photo-inactivation of the nitrogenase since nitrogenase alone shows a lower activity after photoreduction. It seems possible that some protecting factor(s) have been removed by DEAE-cellulose chromatography, since such inactivation by photoreduction is not observed in preparation C_{42}-I (Fig. 3B). On the other hand, factors such as the exact stoichiometry or the time needed to form a tight complex between the components, which have been shown to be highly important (*cf.* [14]), have not yet been studied. Nevertheless, the iron-sulfur protein II has, besides its regulating properties, remarkable protecting effects on the nitrogenase. We observed for instance that after 4 days of storage at room temperature in the absence of dithionite, the reconstituted nitrogenase had lost all activity. The same complex to which iron-sulfur protein II had been added retained 60% of its activity, an observation which confirms those of Haaker and Veeger [14] and Kelly *et al.* [31].

Statements can be found in the literature that iron-sulfur proteins are either contaminants of *Azotobacter* nitrogenase preparations [38] or that some iron-sulfur proteins are actually involved in nitrogenase fixation [39–41]. No claims have been made with respect to iron-sulfur protein II. Nitrogenase and iron-sulfur protein II remain together through a 40-fold purification of the enzyme. That an interaction occurs, is clear from the difference in behaviour during DEAE-cellulose chromatography. Iron-sulfur protein II in the Bulen-Le Comte nitrogenase preparation, is bound relatively tightly to DEAE-cellulose, but the free protein hardly binds at all (*cf.* [13]). Since iron-sulfur protein II is apparently not membrane-bound [13], its interaction with the nitrogenase components is expected to occur also *in vivo*. The effect of iron-sulfur protein II on nitrogenase activity has physiological relevance. Oxygen-protection of the nitrogenase is a major problem of an aerobic cell. *A. vinelandii* nitrogenase has been shown to be protected by Fe-S protein II from inactivation by oxygen [14]. The degree of protection was measured in mixtures of nitrogenase components 1 and 2 plus Fe-S protein II in the absence of reducing agent. In whole *Azotobacter* cells, under conditions of excessive oxy-

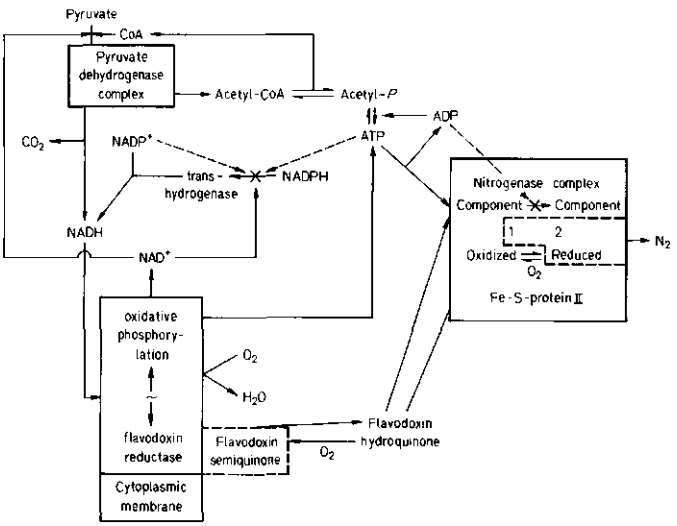

Fig. 6. *Proposed scheme for electron transport from NADH to nitrogenase.* The nitrogenase complex consists of components 1 and 2 and Fe-S protein II. It is assumed that only the reduced form of Fe-S protein II modifies the properties of components 1 and 2

gen supply, a 'switch-off' of nitrogen fixation is observed [42]. It is reasonable that this 'switch-off' condition observed *in vivo* presents a condition of nitrogenase in which it is protected towards oxygen inactivation, which *in vitro* can be induced by Fe-S protein II. This proposal is strengthened by the observation that, with flavodoxin hydroquinone as donor a lag phase becomes apparent in crude preparations, which, in our opinion reflects the conversion of the inactive oxygen-protected 'switch-off' state into the active form. Full activity is regained by reduction, or removal, of the iron-sulfur protein II.

Flavodoxin hydroquinone also could play an important role in the 'switch-off' mechanism (*cf.* [42]). The hydroquinone is very oxygen-sensitive and rapidly oxidized by traces to the semiquinone. Our results indicate that when the redox potential of the semiquinone/hydroquinone couple declines to a value of around -475 to -480 V a 'switch-off' state is induced in the nitrogenase activity, a phenomenon which is dependent on the presence of iron-sulfur protein II. In its absence a normal redox-potential dependency is observed. A similar dependency of the nitrogenase activity in *Clostridium pasteurianum* could be expected since the presence of a red paramagnetic protein similar to Fe-S protein II of *A. vinelandii* has been reported [40,41,43]. Similarly by genetic evidence the need for a protein necessary for the regulation of nitrogenase activity of *Klebsiella pneumoniae* has been proposed [44]. A parallel might also be found in the case of *Rhodospirillum rubrum*, where a factor has been isolated that stimulates nitrogenase activity when collected under N_2 but inhibits when collected aerobically [45]. The question, whether iron-sulfur protein II effects the oxygen-protected state by inducing a certain conformation in the catalytic entities of nitrogenase or by physical protection of oxygen-sensitive sites is under investigation.

Based on the evidence presented here and the data accumulated previously [12,14,16] the following scheme can be postulated for electron transport through the membrane from NADH towards nitrogenase (Fig. 6).

In the scheme it has to be visualised that flavodoxin hydroquinone is produced by membrane energization (*cf.* [14], Fig. 5). Furthermore it was taken into consideration that we have reported [10,46] that *A. vinelandii* contains a phosphotransacetylase in strong interaction with the pyruvate dehydrogenase complex, as well as an acetate kinase. ATP synthesized this way can be coupled to nitrogenase [47]. NADPH formation can be linked to nitrogenase *via* the NADPH-NAD^+ transhydrogenase. This enzyme is strongly inhibited by $NADP^+$ and ATP, while only at a high NADPH/$NADP^+$ ratio formation of NADH takes place [48,49].

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Nitrogen Fixation, Volume I
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III. MEMBRANE ENERGIZATION AND NITROGEN FIXATION IN *AZOTOBACTER VINELANDII* AND *RHIZOBIUM LEGUMINOSARUM.*

C. Veeger, C. Laane, G. Scherings,
L. Matz, H. Haaker, and L. Van Zeeland-Wolbers

Abbreviations: TTFB, 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazol; CCCP, carbonyl cyanide *m*-chlorophenylhydrazon; N_2ase, nitrogenase; BSA, bovine serum albumin; ACMA, 9-amino-6-chloro-2-methoxyacridine; H_2ase, hydrogenase; FeS II, *A. vinelandii* iron-sulfur protein II; Av1, *A. vinelandii* N_2ase protein I; Av2, *A. vinelandii* N_2ase protein II; Ac2, *A. chroococcum* N_2ase protein II.

During the last few years, it has become clear that, in the aerobe *Azotobacter vinelandii*, a number of factors contribute to the complicated process of nitrogen fixation (Haaker, de Kok, and Veeger, 1974; Haaker, Scherings, and Veeger, 1977; Haaker and Veeger, 1977; Scherings, Haaker, and Veeger, 1977; Veeger, Haaker, and Scherings, 1977). It was shown that membrane energization rather than a high ATP:ADP ratio is the determining factor in aerobic nitrogen fixation. Furthermore, we showed that flavodoxin hydroquinone is the major electron donor to N_2ase, although the presence of a NAD(P)H flavodoxin oxidoreductase was also reported. Autooxidation of flavodoxin hydroquinone by O_2 is the major factor that "switches off" (cf. Dalton and Postgate, 1969) nitrogen fixation, whereas N_2ase itself was stabilized toward O_2 inactivation and regulated in activity by the presence of a stoichiometric amount of the pink 2Fe-2S protein

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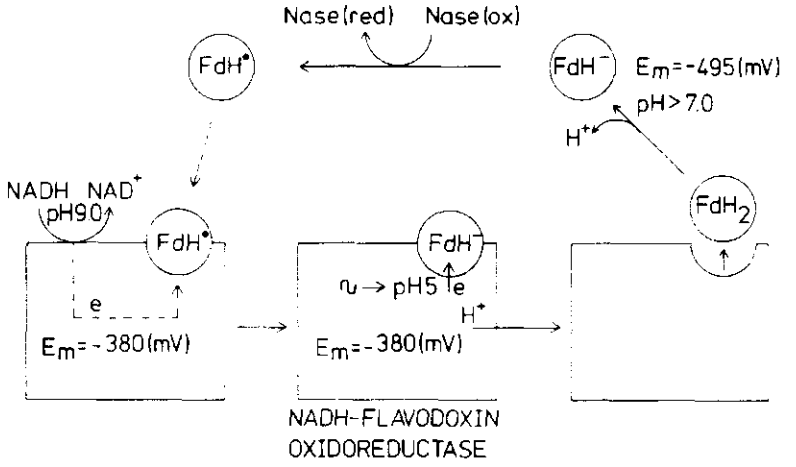


Figure 1. Proposed scheme for electron transport from NADH to N_2ase in *A. vinelandii*.

isolated and characterized earlier (Shethna, DerVartanian, and Beinert, 1968; DerVartanian, Shethna, and Beinert, 1969). In fact, an O_2-stable complex had already been described by Bulen and Le Comte (1972). In the scheme of Figure 1 (cf. Haaker and Veeger, 1977), it was visualized that the proton-driving force over the membrane, as coupled to respiration, lowers the local pH in the vicinity of the binding site of flavodoxin semiquinone to the inner membrane via membrane energization. Under the conditions of a local pH of 4.5–5, the potential of the couple flavodoxin semiquinone/flavodoxin hydroquinone is around −380 mV, low enough to allow reduction by NAD(P)H via a site with pH 8–9. Dissociation of the hydroquinone and subsequent deprotonation in the cytoplasm at pH > 7 decrease the potential (~ −460 mV). The present paper deals with an extended study of the factors mentioned above, both in *A. vinelandii* and *Rhizobium leguminosarum*.

MEMBRANE ENERGIZATION IN RELATION TO NITROGEN FIXATION

Little is known about the supply of reducing equivalents to N_2ase in bacteroids. Appleby, Turner, and MacNicol (1975) noted that the correlation between the ATP:ADP ratio and N_2ase activity in soybean bacteroids was poor when CCCP was used as an uncoupling agent. Although those authors favor a different interpretation, their results suggest that, in addition to the ATP:ADP ratio, the method of generating reducing equivalents is important in supporting N_2ase activity in vivo.

Several techniques have been developed to follow respiration and N_2ase activity in aerobic nitrogen-fixing bacteria (Bergersen, Turner, and Appleby, 1973; Haaker, de Kok, and Veeger, 1974; Wittenberg et al., 1974; Bergersen and Turner, 1975a). The usefulness of the "shaking assay" method for bacteroid suspensions was questioned by Stokes (1975), who deduced mathematically that with hemoprotein present effects other than facilitated O_2 diffusion might occur. Furthermore, the free O_2 concentration cannot be measured and therefore it is not certain whether or not equilibrium conditions exist. Bergersen and Turner (1975a) devised a "no-gas-phase" system in which the free O_2 concentration, the deoxygenation of O_2-binding protein, and acetylene reduction could all be recorded. It is clear that this system cannot achieve the steady-state conditions likely to occur in vivo. We devised a compromise technique that assays simultaneously the steady-state free O_2 concentration and N_2ase activity under conditions of steady-rate respiration (Figure 2) and that takes at least 2 min to reach steady-state conditions (Laane, Haaker, and Veeger, 1978).

The system consists of an 8-ml magnetically stirred, gas-tight vessel with an O_2 electrode located at the bottom (Rank Brothers, Bottisham, Cambridge). Reactants are added by syringe through a rubber stopper. An amplifier is used to detect low O_2 concentrations. The electrode response was calibrated by adding small amounts of air-saturated water to the anaerobic reaction mixture. This system was also used to determine ATP made:O_2 consumed (P:O) ratios in vesicles of *R. leguminosarum* bacteroids and of *A. vinelandii* under conditions of steady-state respiration. The steady-state concentration of free O_2 in the solution is determined by the partial pressure of O_2 in the gas phase, the rate of stirring, and the rate at which O_2 is consumed by the cells or vesicles. The stirring speed and the amount of cells or vesicles were usually held constant and the concentration of free O_2 (c.q. respiration rate) was therefore determined by varying the partial pressure of O_2 in the gas phase. The O_2 input into the solution at the standard stirring speed was calculated by adding known amounts of O_2 to the gas phase and measuring the initial rate of increase of the O_2 tension in the anaerobic liquid phase (Figure 2). Anomalies introduced by the gas-liquid interface were minimized, if necessary, by increasing the amount of liquid so that the surface-to-volume ratio decreased.

This system was used to study the effect of O_2 on the N_2ase activity of *R. leguminosarum* bacteroids under controlled steady-state conditions (Figure 3). Figures 3A and 3B show that the nitrogen-fixing efficiency of freshly prepared *R. leguminosarum* bacteroids can be considerably enhanced by the addition of fatty acid–free BSA. BSA stimulates steady-state N_2ase activity, O_2 consumption, and oxidative phosphorylation without affecting the free O_2 concentration at which maximum acetylene

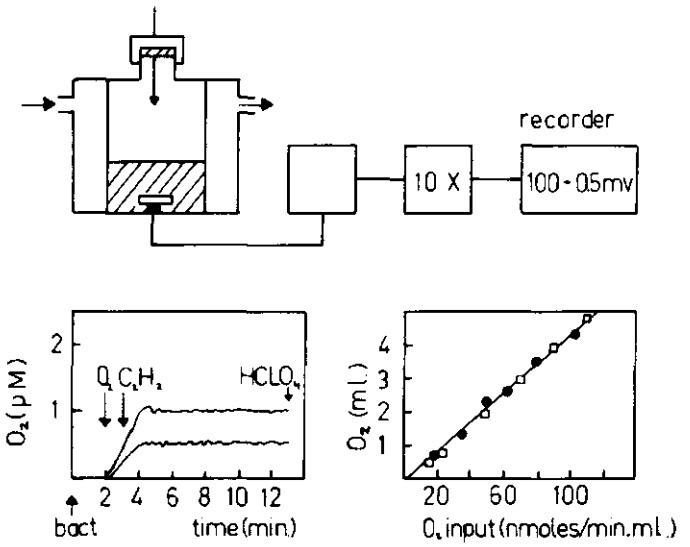


Figure 2. Experimental system (see text and Laane, Haaker, and Veeger, 1978).

formation occurs. At high O_2 concentrations, the ATP:ADP ratio remains fairly constant and the observed decline in nitrogen fixation is therefore not due to a decreased ATP supply as proposed by Bergersen and Turner (1975b) for soybean bacteroids, but rather to inhibition by excess O_2, the so-called switch off state (cf. Haaker, Scherings, and Veeger, 1977; Haaker and Veeger, 1977). This also occurs in the presence of myoglobin and leghemoglobin as O_2 carriers, but even under these conditions fat-free BSA stimulates N_2ase activity about twofold without influencing the ATP:ADP ratio. We thus conclude that nitrogen fixation in *Rhizobium* bacteroids, as in *A. vinelandii* cells, is dependent mainly on the state of energization of its membranes and to a lesser extent on the ATP:ADP ratio. During the preparation of bacteroids, the cell membrane is exposed to the uncoupling effect of free fatty acids and to plant phospholipase D activity (Laane, Haaker, and Veeger 1978). Both effects can be counteracted by BSA provided that the fatty acid-free form is used (Table 1).

Figure 4 demonstrates the effect of the uncoupler CCCP on the N_2ase activity and the ATP:ADP ratio of *R. leguminosarum* bacteroids. CCCP strongly inhibits N_2ase activity of bacteroids with little or no effect on the ATP:ADP ratio (Figure 4A). At concentrations higher than 1 μM, an approximately linear relationship exists between ATP:ADP ratio and N_2ase activity (Figure 4B). Without uncouplers, however, the relation, obtained under conditions at which O_2 concentrations were lower than necessary to obtain maximum N_2ase activity, is also linear but with a different slope.

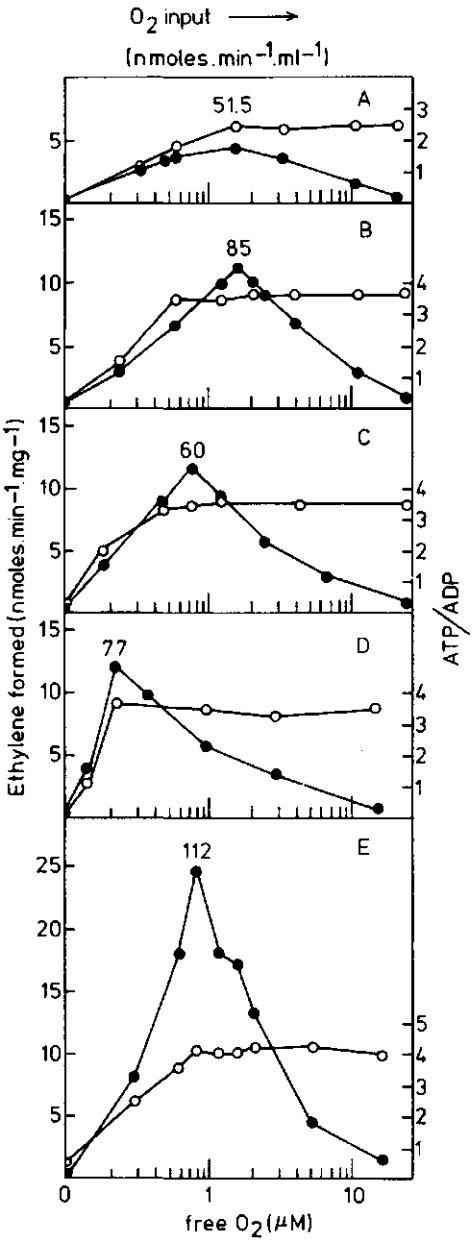


Figure 3. The relationship between free dissolved O_2 concentration, O_2 consumption, N_2ase activity and ATP:ADP ratio of bacteroids as influenced by different effectors (cf. Laane, Haaker, and Veeger, 1978). ●——●, N_2ase; ○——○, ATP:ADP. A, without addition; B, 3.1% BSA; C, 130 μM myoglobin; D, 110 μM leghemoglobin; E, 130 μM myoglobin plus 3.1% BSA.

Table 1. Effect of linoleic acid on N_2ase activity and ATP:ADP ratio of *R. leguminosarum* bacteroids (see Laane, Haaker, and Veeger, 1978)

Linoleic acid (μg)	Addition	C_2H_4 evolution (nmol min^{-1} mg^{-1})	ATP:ADP
—	—	12	3.5
—	C_2H_5OH	12	3.5
18	—	11.3	3.2
36	—	7.7	1.6
54	—	3.5	1.0
36	3% BSA	11.6	3.4

When ACMA is used as a fluorescent probe, a lower energized state of the membrane is observed at all CCCP concentrations used (cf. Haaker, de Kok, and Veeger, 1974). Because the only known effect of CCCP is to lower the energized state of the membrane, these results also show that, as well as being controlled by the ATP:ADP ratio, N_2ase activity is determined by the supply of reducing equivalents, which itself is related to the state of membrane energization.

Studies with oligomycin excluded the possibility that the decline of N_2ase activity was due to decreased ATP synthesis and a lowered utilization of ATP by N_2ase (Veeger, Haaker, and Scherings, 1977). Oligomycin lowers the rate of ATP synthesis but does not affect N_2ase activity. Appleby, Turner, and MacNicol (1975) also noted that the relation between the ATP:ADP ratio and N_2ase activity was poor when CCCP was used. Their explanation that separate domains of ATP formation and accumula-

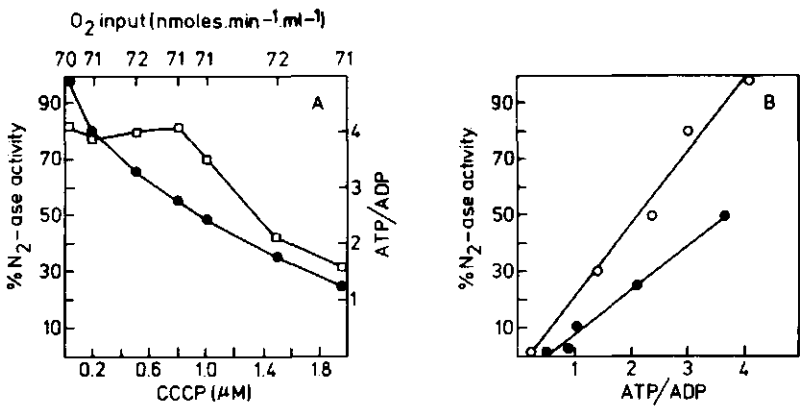


Figure 4. A) Influence of CCCP on O_2 consumption, N_2ase activity and ATP:ADP in *R. leguminosarum* bacteroids. (●——●), N_2ase; (□——□), ATP:ADP. B) Relationship between bacteroid N_2ase activity and ATP:ADP in the presence (●——●) and absence (○——○) of CCCP (cf. Laane, Haaker, and Veeger, 1978).

tion exist within the bacteroid and that one of these domains is more sensitive to uncoupling seems very unlikely. Our results show that their data can also be explained in terms of limitation in electron supply to N_2ase.

We have tried to induce N_2ase activity in an in vitro system in which the reducing equivalents are generated by membrane vesicles from either *A. vinelandii* cells or *R. leguminosarum* bacteroids. For this purpose, flavodoxin, which is a good electron donor for N_2ase in the hydroquinone state (Scherings, Haaker, and Veeger, 1977), and N_2ase complex (Scherings, Haaker, and Veeger, 1977), both purified from *A. vinelandii*, were added to the vesicle suspension. Although we have been successful in preparing vesicles that can be highly energized by respiration (Figure 5), attempts to reduce flavodoxin to the hydroquinone state by an energized membrane were unsuccessful. One major problem could be the failure to energize these vesicles directly by ATP, because flavodoxin hydroquinone is readily oxidized to the semiquinone state by O_2. Treatment of the vesi-

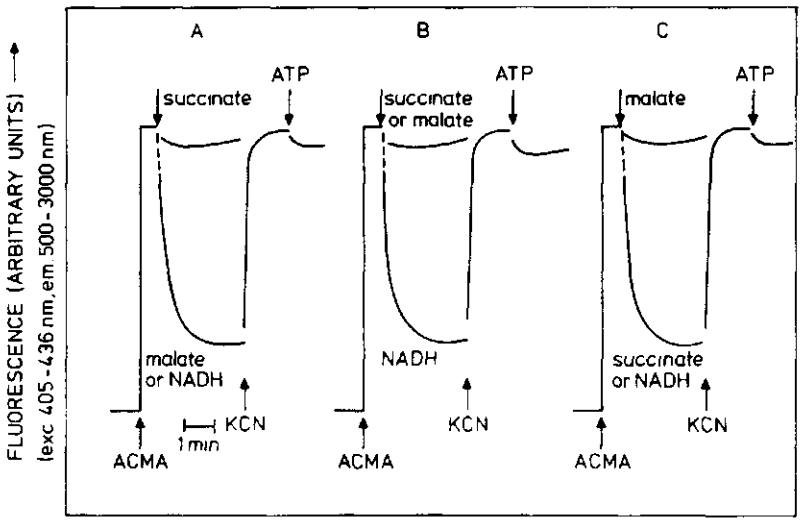


Figure 5. Quenching of ACMA fluorescence by $NaCl^-$ and $NaCl^-$ membrane vesicles of *A. vinelandii* and *R. leguminosarum* bacteroids on energization. Vesicles of *A. vinelandii* were isolated anaerobically from cells harvested at the turnover from logarithmic into nonlogarithmic growth (A_{650} = 0.9–1.0) by sonication of lysozyme-EDTA-treated cells. The membranes were sedimented by centrifugation between 18,000 and 100,000 × *g*. Bacteroids from pea root nodules were isolated as described by Bergersen (1971) and its vesicles as for *A. vinelandii*. Vesicles were washed and suspended in a medium containing 50 mM Tes/KOH, 4 mM $MgCl_2$, and 0.33 M NaCl where indicated (pH 7.6). ACMA (2 μM), succinate (10 mM), malate (10 mM), NADH (0.25 mM), KCN (5 mM), and ATP (1 mM) were added at the times indicated. Membrane vesicles were added to a final concentration of 0.02 mg of protein/ml. Data are corrected for NADH fluorescence. (A). $NaCl^-$ and $NaCl^-$ membrane vesicles of *A. vinelandii*; (B). $NaCl^-$ membrane vesicles of *R. leguminosarum* bacteroids; (C). $NaCl^-$ membrane vesicles of *R. leguminosarum* bacteroids.

cles with trypsin (Bhattacharyya and Barnes, 1976) or by column chromatography (Racker and Horstman, 1967) to remove the ATPase inhibitor is ineffective. Furthermore, Figure 5 shows that there are major differences between the two types of vesicles. Vesicles from *A. vinelandii* cells can easily be energized by oxidation of malate and NADH, but barely with succinate both in the presence and absence of NaCl. On the other hand, in the absence of NaCl, vesicles from *R. leguminosarum* bacteroids can only be energized by NADH oxidation and not by oxidation of succinate or malate. However, when the vesicles were made in the presence of NaCl ($NaCl^+$), succinate oxidation could induce energization to the same extent as NADH oxidation, whereas malate still was hardly active.

It should be noted that soybean bacteroids also contain considerable amounts of flavodoxin (Phillips et al., 1973). Thus, in bacteroids reducing equivalents are likely to be donated by flavodoxin hydroquinone to the N_2ase, a postulate made probable by the high activities observed with bacteroidal N_2ase in the presence of *A. vinelandii* flavodoxin hydroquinone (Table 2). Furthermore, we could demonstrate the presence of a NADH-flavodoxin reductase in extracts of bacteroids.

The presence of a unidirectional, H_2-oxidizing H_2ase in a number of nitrogen-fixing organisms and its relation with nitrogen fixation have been well established (Wilson and Burris, 1947; Lindström, Lewis, and Pinsky, 1951; Dixon, 1976; Schubert and Evans, 1976; Smith, Hill, and Yates, 1976; Kelley et al., 1977). This H_2ase enables these organisms to recover some of the energy lost during wasteful production of H_2 by N_2ase, resulting in a higher nitrogen-fixing efficiency. We have studied membrane energization and oxidative phosphorylation connected with H_2 oxidation in membrane vesicles of *A. vinelandii* and *R. leguminosarum*. Figure 6A shows that membrane vesicles of *A. vinelandii* can be energized by H_2 as an electron donor. Figure 6B clearly shows no flavoprotein involvement in the respira-

Table 2. Comparison of the efficiency of several electron donors for N_2ase activity in a crude extract of *R. leguminosarum* bacteroids (strain PRE)[a]

Donor(s)	%
Dithionite	100
Flavodoxin	75
Ferredoxin I	58
Flavodoxin + ferredoxin I	127

[a] Bacteroids were isolated as described in Laane, Haaker, and Veeger (1978). A crude extract was prepared by sonicating the bacteroids suspension for 2 min and centrifuging the ruptured cells for 1 hr at 38,000 × *g*. Flavodoxin and ferredoxin I purified from *A. vinelandii* and reduced by photoreduction with deazaflavin according to Scherings, Haaker, and Veeger (1977) were added to a final concentration of 10 μM. In a standard assay 0.4 mg of crude extract protein was added to the incubation mixture. Data are expressed as percentage of acetylene reduction rate with dithionite as electron donor.

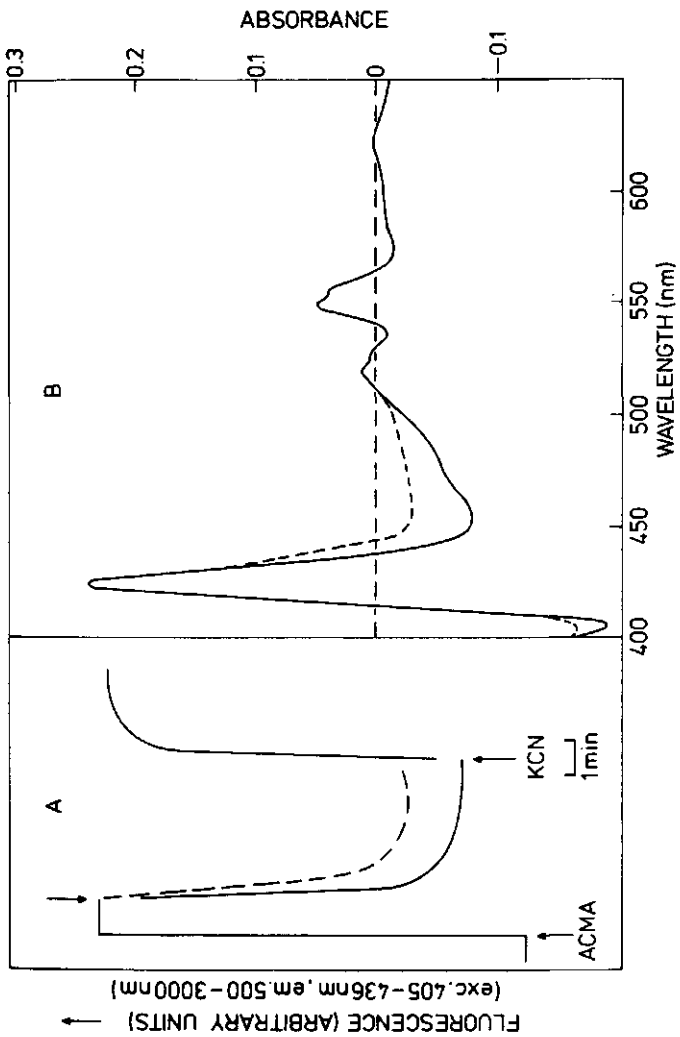


Figure 6. Oxidation of H_2 by membranes of *A. vinelandii*. (A) Quenching of ACMA fluorescence by membrane vesicles with NADH and H_2. Vesicles were isolated as in Figure 5 and suspended in a medium containing 50 mM Tes/KOH and 4 mM $MgCl_2$ (pH 7.4). ACMA, NADH, and KCN were added to a final concentration of 2 μM, 0.3 mM, and 5 mM, respectively, at the times indicated by arrows. In a separate experiment (----) hydrogen (50%) was added to the gas phase of a closed cuvette that was shaken at the time indicated. 0.02 mg of vesicle protein was used for each assay. (B) Reduced minus oxidized difference spectra of membrane vesicles. Vesicles were suspended in a medium containing 50 mM Tes/KOH and 2.5 mM $MgCl_2$ (pH 7.0) to a final concentration of 3.4 mg of protein per ml. NADH (——) was added to a final concentration of 0.6 mM; hydrogen (----) was added as described in A.

tory chain of *A. vinelandii* when H_2 is used as substrate. Vesicles of *R. leguminosarum* (strain PRE) did not oxidize H_2 and therefore failed to induce membrane energization.

P:O RATIOS IN MEMBRANE VESICLES OF *A. VINELANDII* AND *R. LEGUMINOSARUM* BACTEROIDS

Assessment of the efficiency of oxidative phosphorylation in intact cells and isolated respiratory membranes of *A. vinelandii* has been attempted (Ackrell and Jones, 1971; Baak and Postma, 1971; Eilermann et al., 1971; Haaker and Veeger, 1976). The P:O values reported for intact cells of *A. vinelandii* (Baak and Postma, 1971) suggest a complete coupling between oxidation and phosphorylation, whereas isolated respiratory membranes are much less efficient (Ackrell and Jones, 1971; Haaker and Veeger, 1976). No P:O values are reported for *R. leguminosarum* bacteroids. We decided to study the oxidative phosphorylation efficiency in membrane vesicles of *A. vinelandii* and *R. leguminosarum* in the assay system described above, which allows investigation of this parameter under controlled steady-state respiration rates. With a suspension of *A. vinelandii* vesicles and NADH as substrate, Figure 7 shows that when the O_2 supply is increased the P:O ratio rises to a maximum, but declines when O_2 becomes detectable in the medium. At the same time, the ATP concentration rises to a fairly constant level as the O_2 supply is increased. The shape of the curve clearly demonstrates that a certain amount of energization of the membrane is necessary for full ATP synthesis. Similar phenomena are observed with malate or H_2 as substrates, except that at high O_2 input the ATP concentration declines rapidly as H_2 is used as a substrate. This decline appears not to be caused by a limited amount of H_2, but to inhibition of H_2ase by excess O_2. The maximum oxidation capacities are not completely reached at the highest P:O ratio. The calculated P:O values are based on real oxidation rates and are therefore corrected for the appearance of O_2 in the medium. The maximum P:O ratio obtained with H_2 is comparable with that obtained with NADH. Furthermore, addition of acetylene to the gas phase (final concentration 20%) does not inhibit H_2 respiration. Exactly the same curves are obtained as those presented in Figure 7C, which contrasts with the previous results obtained with *Azotobacter chroococcum* (Smith, Hill, and Yates, 1976).

Figure 8 shows the results obtained with vesicles of *R. leguminosarum* bacteroids. Although higher P:O ratios are observed with NADH as substrate, the general concept is similar to that of *A. vinelandii*. Also shown is the effect of fatty acid–free BSA on the efficiency of the oxidative phosphorylation, which supports the conclusion that the cytoplasmic membrane of *R. leguminosarum* bacteroids is partially uncoupled by free fatty acids.

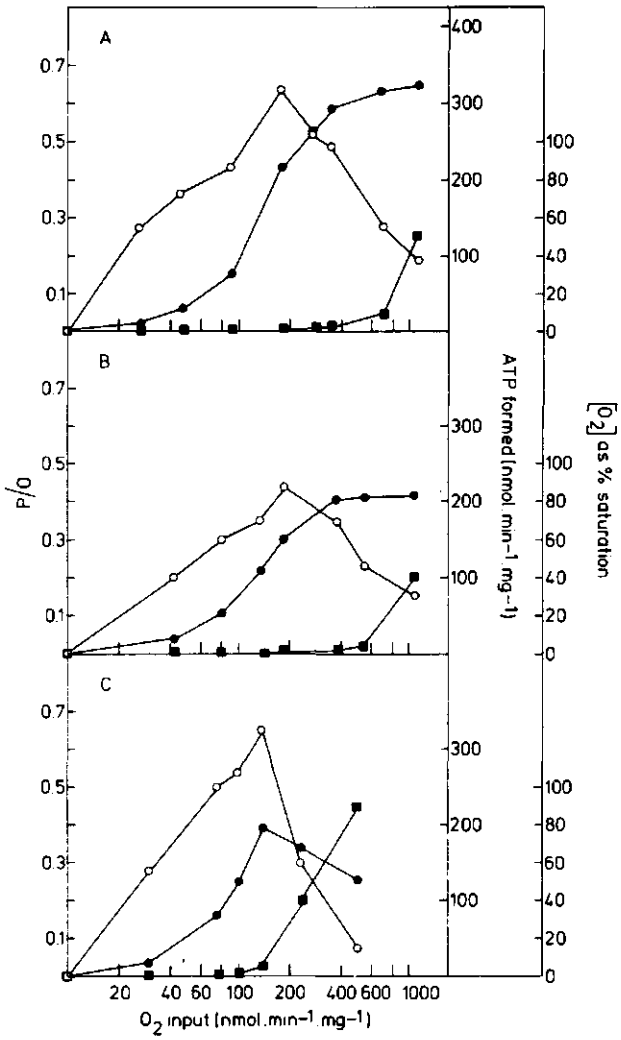


Figure 7. Effect of O_2 on P:O ratio and ATP synthesis in membrane vesicles of *A. vinelandii*. Vesicles were isolated as in Figure 5 and suspended and stored at 0°C in a medium containing 50 mM Tes/KOH, 2.5 mM $MgCl_2$, and 1.2% fatty acid-free BSA (pH 7.0). Experiments were performed with the system described in the text. The standard incubation mixture contained at 30°C: 50 mM Tes/KOH, 2.5 mM $MgCl_2$, 10 mM glucose, and 5 mM phosphate (final pH 7.0). The incubation mixture, total volume 2.5 ml, and the reaction vessel were made anaerobic by flushing with argon. Three minutes after addition of 15 U of a dialyzed hexokinase solution, vesicles (0.35 mg protein), substrate, and variable amounts of O_2 were added to the gas phase. After 3 min stirring, when the system was found to be in equilibrium, an anaerobic solution of ADP (final concentration 0.5 mM) was added. After 2.5 min incubation with ADP, the vesicles were rapidly fixed with $HClO_4$ up to a final concentration of 4% (w/v) and the level of glucose 6-phosphate was determined (Haaker, de Kok, and Veeger, 1974). A logarithmic scale indicates the O_2 input. It is plotted against: (O——O), P:O ratio; (●——●), ATP synthesis; (■——■), O_2 concentration expressed as percentage saturation. (A), NADH, final concentration 0.6 mM; (B), malate, final concentration 5 mM; (C), H_2, 50% in the gas phase.

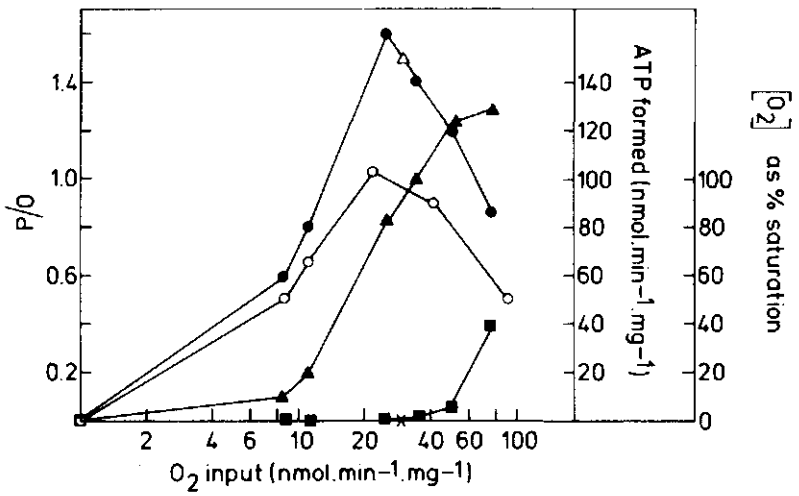


Figure 8. Effect of O_2 on P:O ratio and ATP synthesis in membrane vesicles of *R. leguminosarum* bacteroids. Bacteroids from pea root nodules were isolated as earlier described (Bergersen, 1971). Vesicles were prepared as described in Figure 5 for *A. vinelandii*, except that 0.3 M sucrose was present during the isolation procedure, and that BSA was omitted in the final suspension. When indicated fatty acid-free BSA (final concentration 2.5%) was added to the incubation mixture. Assay conditions as in Figure 7, except that 2.4 mg of vesicle protein were added to the incubation mixture; final volume 5 ml, temperature 25°. O_2 input is plotted against: (○——○), P:O ratio with NADH; (●——●), P:O ratio with NADH and BSA; (△), P:O ratio with NADH and BSA in an incubation volume of 4 ml; (X), P:O ratio with succinate (final concentration 5mM); (▲——▲) ATP synthesis with NADH; (■——■), O_2 concentration expressed as percentage saturation.

Furthermore, the respiration rate of *R. leguminosarum* with NADH is about eighteen times less than that of *A. vinelandii* vesicles. Although succinate is rapidly oxidized by vesicles of *R. leguminosarum* bacteroids, no significant ATP synthesis could be detected, which means that succinate oxidation is uncoupled in these $NaCl^-$ vesicles (cf. Figure 5B). Energization of $NaCl^+$ membranes, as induced by succinate oxidation (cf. Figure 5), is due to coupled oxidative phosphorylation (ATP synthesis) under these conditions (not shown in Figure 8). The reason for this difference is not known yet, but it is possibly due to extraction of an essential component, because we observed some ATP formation in poorly washed $NaCl^-$ vesicles. H_2 is not oxidized at all and O_2 appears immediately in the medium with both $NaCl^-$ and $NaCl^+$ vesicles. We therefore conclude that there is no active H_2ase present in both types of vesicles. The P:O ratio with NADH, determined in a 4-ml or 5-ml assay mixture, is exactly the same, which excludes the possibility that gas-liquid phase interactions interfere with this determination.

NAD(P)H-FLAVODOXIN OXIDOREDUCTASE

A reductase that reacts with flavodoxin from *A. vinelandii* in the presence of NAD(P)H was purified (200 times) until it gave one band on SDS polyacrylamide gel electrophoresis (Table 3). Yates (1971) has described a NADH dehydrogenase that was purified only 10–15 times with respect to the benzyl viologen reductase activity. We made a similar observation with our enzyme (purification 25–30 times). The major difference is that our enzyme catalyzes flavodoxin reduction by NAD(P)H and that this activity is purified about 200 times. Nevertheless, the specific activity of our purest preparations is not very high, and varies between 6 and 30 $nmoles \cdot min^{-1} \cdot mg^{-1}$. We believe it to be a flavodoxin reductase because of the spectrophotometric differences occurring at 450 nm and 615 nm on reduction of flavodoxin by NADH. If the amount of flavodoxin semiquinone (as measured by the increase in absorbance at 615 nm) is calculated and compared with the value calculated from the decrease in absorbance at 450 nm, the latter value is much larger. This difference is explained by formation of hydroquinone (no 615-nm absorption) in addition to semiquinone. After proper correction, we calculate that the ratio of hydroquinone formed to semiquinone formed varies between 0.5 and 1.5 in different preparations. From the point of view of flavoprotein catalysis (see below) two-electron transfer (Blankenhorn, 1977; Hemmerich, 1977), and

Table 3. Purification of flavodoxin reductase[a]

Preparation	Total volume (ml)	Total protein (mg)	Specific activity	Purification	Yield
Crude supernatant	212	4092	0.029	1.0	100
Protamine sulfate supernatant	262	2019	0.049	1.8	88.7
DEAE cellulose pool	250	500	0.23	82	105.9
Hydroxylapatite pool	6.6	12.2	2.1	75.0	23.2
Sucrose density gradient centrifugation	23.1	5.9	6.1	218.0	32.4

[a] *A. vinelandii* cells (114 g) that had been frozen and stored at −20°C were broken in a french pressure cell by two passes through the cell in 50 mM Tes buffer (pH 7.4). After removal of the cellular debris by centrifugation at 15,000 × *g* for 0.5 hr, the membranes were removed by ultracentrifugation at 250,000 × *g* for 4 hr. The crude supernatant was treated with 0.1 mg protamine sulfate/mg of protein. The protamine sulfate supernatant was dialyzed against 5 mM Tes and applied to DEAE cellulose. The column was washed with 5 mM Tes (pH 7.4). After elution with 0.1–0.5 M NaCl in 5 mM Tes, the enzyme was applied to hydroxylapatite and then was eluted from it by between 0.1 and 0.3 M potassium phosphate buffer (pH 7.4). This pool was then layered on a 0%–20% sucrose (w/w) gradient and centrifuged at 100,000 × *g* for 4 hr. The initial absorbance increase at 615 nm was registered (ϵ = 5.3 mM cm^{-1}); specific activity expressed as $nmoles\ mg^{-1}\ min^{-1}$.

thus hydroquinone formation, is more likely to occur. Assuming two-electron reduction of the flavodoxin (the E_m for the quinone/hydroquinone couple of -270 mV does not exclude this), the formation of semiquinone thus seems to be caused by comproportionation of the hydroquinone formed with unreacted quinone.

The system of flavodoxin reduction by NAD(P)H is complicated by two phenomena:

1. The rate of reduction is only linear in a very limited range of enzyme concentrations. A tenfold increase in enzyme concentration leads to strong (50%–60%) inhibition. We have no explanation to offer yet.
2. The tracings of flavodoxin reduction at either 615 nm or 450 nm are nonlinear. With time, an activation of the rate of reduction at both wavelengths is observed. This is not due to substrate inhibition by flavodoxin since, for practical reasons, we are assaying at flavodoxin concentrations of 20–30 μM, which is below the K_m value of *A. vinelandii* flavodoxin ($\sim$100 μM). However, the reaction with benzyl viologen as acceptor is linear with enzyme concentration. The pH profile of the flavodoxin reductase shows a maximum around 9 (Tris and glycine buffer), whereas the benzyl viologen reductase shows an increase in activity until pH 11 and in this respect resembles the pH profile of NADH dehydrogenase from *A. chroococcum* (Yates, 1971).

In addition to reacting with benzyl viologen, the enzyme reacts with flavodoxins from *A. vinelandii*, *Megasphaera elsdenii*, and *Desulfovibrio vulgaris* under anaerobic condition. The flavodoxins vary in reactivity—*M. elsdenii* > *D. vulgaris* > *A. vinelandii*, an order that corresponds to the redox potentials of the semiquinone-hydroquinone couple. The rates of the *M. elsdenii* and *D. vulgaris* flavodoxins also increase with time. The K_m values of these flavodoxins are lower, however (10–20 μM).

The absorption spectrum and the fluorescence excitation spectrum of this enzyme show maxima at 380 nm and 450 nm (shoulder at 480 nm). The enzyme contains FAD, which is fluorescent. The lifetime of the fluorescence emission of the oxidized flavin, excited at 450 nm, is 1.0 nsec and the degree of polarization of the emission on excitation at the same wavelength is 0.39. Titration of the reductase with NAD(P)H decreases the relative fluorescence of the enzyme while increasing the lifetime of the flavin. The enzyme-NADH complex regains its relative fluorescence on the addition of benzyl viologen. The flavin is quenched by irradiation with deazaflavin-EDTA. Addition of flavodoxin quickly restores the fluorescence.

The enzyme that we isolated resembles in many respects the NADH dehydrogenase isolated by Yates (1971). It is very easy, because of the low turnover under practical assay conditions (20–30 μM flavodoxin), to overlook the flavodoxin reductase activity. Flavodoxin hydroquinone could be

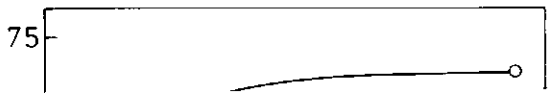
produced at a slow rate without the need for membrane energy. It is known to be rapidly oxidized by N_2ase to the semiquinone (Haaker, Scherings, and Veeger, 1977; Haaker and Veeger, 1977; Scherings, Haaker, and Veeger, 1977; Veeger, Haaker, and Scherings, 1977). Assuming that these processes occur, the problem arises as to how to convert the semiquinone into the hydroquinone, a process that cannot be achieved by NAD(P)H alone. In view of the role of membrane energization in N_2ase activity of both *A. vinelandii* and *R. leguminosarum*, as well as the role of flavodoxin as electron donor in this process, our proposal that the membrane energy is the driving force for flavodoxin hydroquinone formation still appears very attractive and acceptable.

PROTECTION OF *A. VINELANDII* N_2ASE AGAINST DAMAGE BY O_2

Various aspects of O_2 tolerance in nitrogen-fixing organisms have been reviewed (Yates, 1977). Two hypotheses for O_2 tolerance of *Azotobacter* cell-free extracts have been put forward in the past: 1) N_2ase is located in a specialized particle or "azotophore," which provides a physical barrier to O_2 diffusion (Reed, Toia, and Raveed, 1974); and 2) association of N_2ase with membranes or "factors" induces an O_2-tolerant conformation of N_2ase.

The evidence for the first theory is very scarce and Haaker and Veeger (1977) could not find any evidence for localization of N_2ase in a subcellular particle. The second theory is more than an idea because removal of a factor during purification resulted in the transition from O_2 tolerant to O_2 sensitive (Haaker, Scherings, and Veeger, 1977). Haaker and Veeger (1977) subsequently did the appropriate isolation and reconstitution experiment that showed that the isolated factor gave protection toward O_2 on addition to the O_2-sensitive N_2ase components. Scherings, Haaker, and Veeger (1977) identified the factor as FeS II, previously purified and partly characterized by Beinert and co-workers (Shethna, DerVartanian, and Beinert, 1968; DerVartanian, Shethna, and Beinert, 1969). FeS II is a 2Fe-2S protein with a native molecular weight of 24,000 and a midpoint potential of -225 mV (G. Scherings, unpublished data) to -230 mV (Ke et al., 1974). The protein seems to contain more than one peptide chain (G. Scherings, unpublished data). Bulen and Le Comte (1972) reported that FeS II was a small contamination in their preparations of *Azotobacter* N_2ase. We now wish to report in some detail work carried out in part in collaboration with Dr. M. G. Yates on the protection characteristics using purified proteins.

Figure 9 shows the protection of Av1 + Av2 (incubated simultaneously) by FeS II. The activity that remains after 15 and 45 min incubation in the presence of 340 μM O_2 at room temperature is plotted versus the molar ratio of FeS II to Av2. Saturation behavior is observed. A phenomenon for



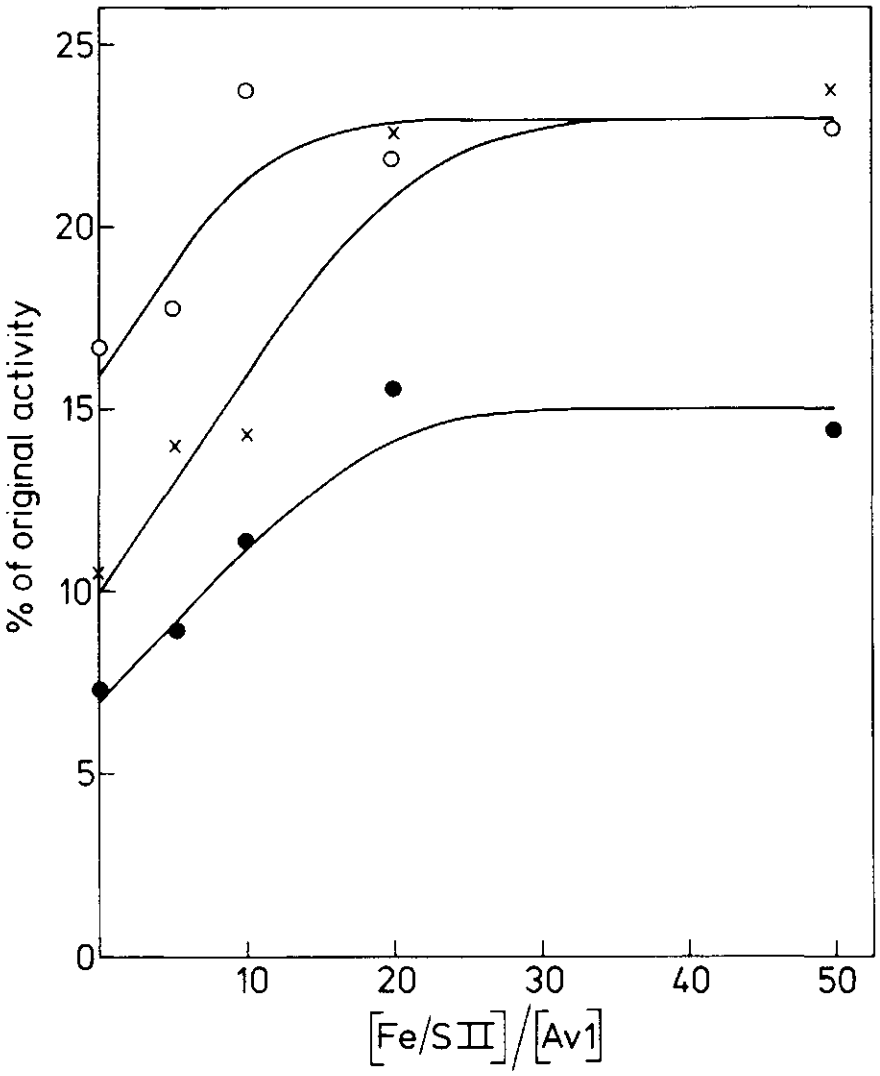


Figure 12. Protection of Av1 by feS II and $MgCl_2$. Inactivation was carried out as described in Figure 9 except that no Av2 was present. The final Av1 concentration was 1.5 μM. Reaction was for 170 min with 400 μM O_2. $MgCl_2$ concentrations: (●——●), none; (X——X), 3 mM; (○——○), 9 mM. Assay for remaining activity was started by injecting 300 μl into the assay mixture described in Figure 9, plus Av2 (twentyfold excess over total Av1). Experimental points represent the average of two activity measurements.

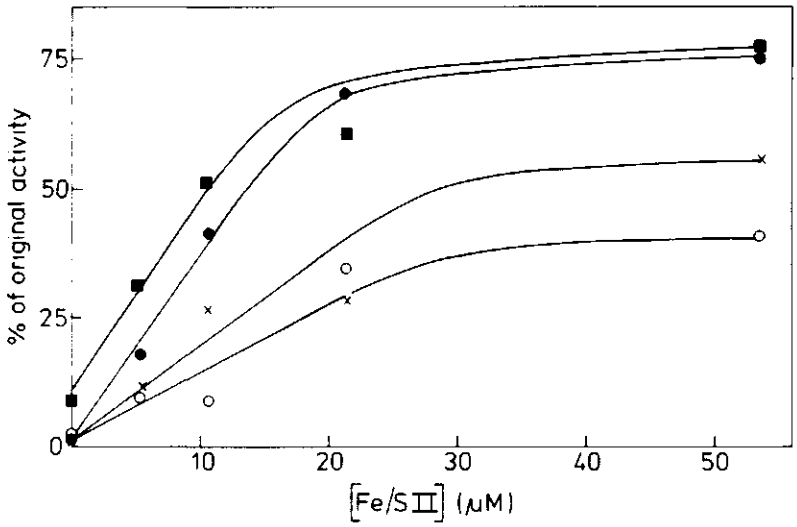


Figure 13. Protection of Av2 by FeS II. Anaerobic mixtures of $Av2_{ox}$ and FeS II_{ox} were prepared in 25 mM Hepes-KOH (pH 7.2) and then diluted twofold in all-glass Chance syringes with 25 mM Hepes-KOH (pH 7.2) that had been sparged with an argon-oxygen mixture to $[O_2]$ = 150 μM. Mixing was done as described in Figure 9. Final concentration of $Av2_{ox}$ was 5.3 μM (●——●) and 13.5 μM (■——■). Final concentrations of FeS II as indicated. Inactivation time was 5 min. The two lower lines were obtained in the presence of either 5 mM $MgCl_2$ (X——X) or 5 mM $CaCl_2$ (O——O) at an Av2 concentration of 5.3 μM. Assay for remaining activity was started by injecting 0.25 (5.3 μM) or 0.10 (13.3 μM) ml into the assay mixture (see Figure 9) plus a fixed amount of Av1 (1.5 times Av2 concentration). Note: The often quoted optimum in activity that is observed when fixed amounts of Av2 are mixed with varying amounts of Av1 might indicate a need for titration in order to estimate correctly the remaining concentration of active Av2. However, in our conditions, this does not appear to be neccessary since the initial linear dependence of C_2H_2-reducing activity on the [Av2] in the presence of a fixed amount of Av1 (linear up to nearly equimolar concentrations) can actually be used as a "standard curve."

and FeS II, stabilized by the divalent cation Mg^{2+}. As judged from the inception of nitrogenase activity of this three-component complex in its reaction with flavodoxin hydroquinone (cf. Scherings, Haaker, and Veeger, 1977), this O_2-stable complex is probably inactive, or at least less active. "Switch on" to the active form is accomplished by an adequate supply of reducing equivalents via flavodoxin hydroquinone. Reduction leads to dissociation of the complex and thus to activation. Such a process can only offer a partial explanation for the rapid "switch off/switch on," O_2-dependent phenomenon observed in several aerobes (Hill, Drozd, and Postgate, 1972). The rapid autooxidation of flavodoxin hydroquinone, which cuts off electron supply, is at least an equally contributing factor in the deactivation of nitrogen fixation in our opinion. However, under such

conditions, the O_2-stable oxidized Av1-Av2-FeS II complex is rapidly formed.

This view is supported by experiments carried out with Dr. R. N. F. Thorneley that indicate that no complex exists between Av2 and FeS II. In studying the effect of Av2 on the reduction of FeS II by $S_2O_4^{2-}$ by stopped-flow spectrophotometry, we observed that reduced Av2 could, at low dithionite concentrations, compete quite effectively with SO_2^- in the reduction of FeS II. Reduction of FeS II by dithionite in the absence of Av2 follows the rate expression $v = 1.6 \times 10^5\ [SO_2^-][\text{FeS II}]\ \text{M}\cdot\text{sec}^{-1}$ (Figure 14). The second-order rate constant is similar to those for other FeS proteins (Lambeth and Palmer, 1973). However, in the presence of Av2 a rate expression of the form $k_{obs} = k_2 + k_3\ [S_2O_4^{2-}]^{1/2}$ is obtained. Since Av2 is reduced rapidly by SO_2^- ($k > 10^8\ \text{M}^{-1}\cdot\text{sec}^{-1}$), like $Ac2_{ox}$ (Thorneley, Yates, and Lowe, 1976), k_2 is interpreted as the rate constant associated with the reduction of FeS II via Av2.

We sought to determine a stability constant for the presumed Av2-FeS II complex involved in the electron transfer reaction. Because the reduction of $Av2_{ox}$ by SO_2^- is much faster than the reduction of FeS II_{ox} at low dithionite concentrations, we rapidly mixed Av2 in the presence of a low di-

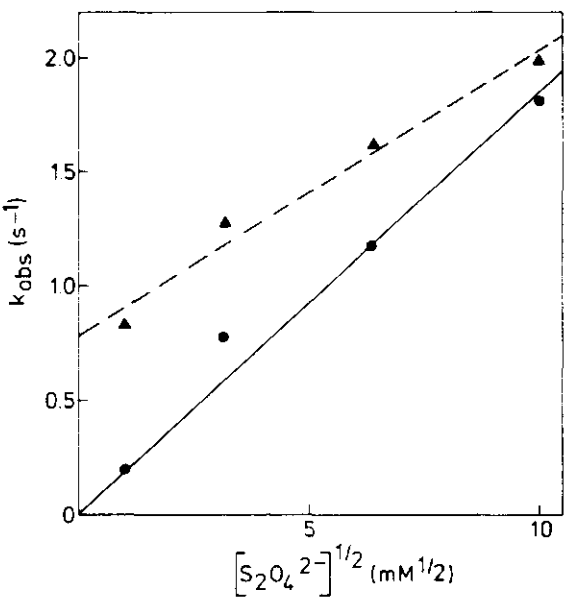


Figure 14. Dependence of the observed rate constant for FeS II reduction on dithionite concentration in the absence and presence of Av2. Final concentrations were FeS II, 44 μM, and Av2, 22 μM. Plotted are FeS II versus $S_2O_4^-$ (●——●) and FeS II + Av2 versus $S_2O_4^-$ (▲——▲).

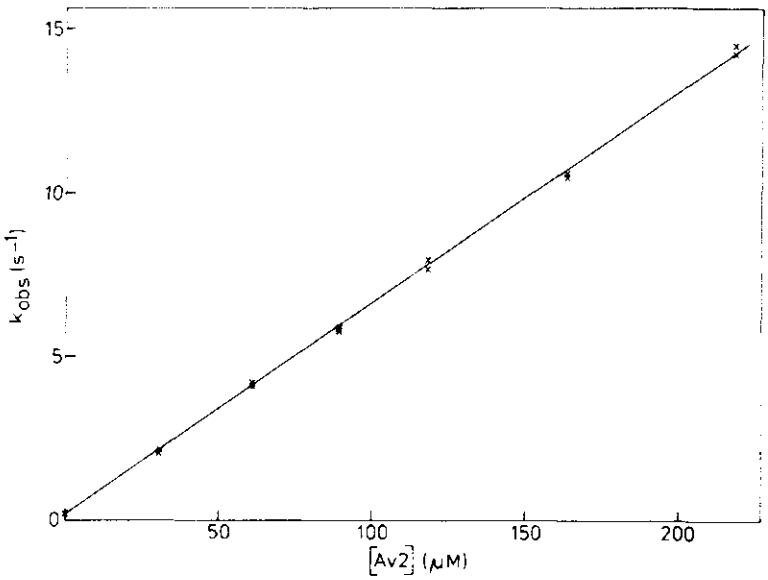


Figure 15. Dependence of the observed rate constant for FeS II reduction on the concentration of Av2 in the presence of a low dithionite concentration. Av2 plus $S_2O_4^-$ was mixed with FeS II. Final Av2 concentrations as indicated; final FeS II concentration was 18 μM, final dithionite concentration 1 mM.

thionite concentration with FeS II_{ox} in the stopped-flow apparatus and observed a second-order rate of reduction of FeS II_{ox} by $Av2_{red}$. Figure 15 shows the dependence of k_{obs} on Av2 concentration at constant dithionite concentration. The plot is not detectably curved up to the highest concentration of Av2 (218 μM) employed. Thus, there is no evidence for complex formation under these conditions. An estimate of the minimum curvature that would have been detected by the experimental system indicates a lower limit of 1 mM for the dissociation constant of the $Av2_{red}$ plus FeS II_{ox} complex. The rate constant for electron transfer between these two iron-sulfur proteins is calculated to be $6.5 \times 10^4\ M^{-1} \cdot sec^{-1}$.

There is good evidence, then, to show that O_2 protection in whole cells and in cell-free and purified extracts of *A. vinelandii* is brought about by the redox-dependent complex formation of Av1, Av2, and FeS II. "Switch on" and "switch off" in whole cells can be explained by our hypothesis; specifically, rapid "switch on" can occur by the rapid reduction of FeS II by Av2. FeS II also protects *A. chroococcum* N_2ase, but not cell-free extracts of *R. leguminosarum* N_2ase, against O_2 inactivation. Thus, *R. leguminosarum* N_2ase probably does not contain a FeS II-type of protein, but may be well protected against inactivation by free O_2 by the high concentration of the leghemoglobin around the bacteroid.

CONCLUSIONS

The results presented here show that, as in *A. vinelandii*, nitrogen fixation in aerobic *R. leguminosarum* bacteroids can only be achieved by energization of the cytoplasmic membrane by means of oxidation energy. The substrates are slightly different, e.g., NADH and malate in *A. vinelandii* and NADH in *R. leguminosarum*. Succinate oxidation energizes the membrane of *A. vinelandii*, and can also energize the membrane of *R. leguminosarum* when these membranes are prepared at high salt concentration. Because succinate oxidase activity is also present at low NaCl concentration, we conclude that, in *R. leguminosarum* membranes, this process proceeds in a non-energy-linked way under these conditions.

Hydrogen oxidation coupled to oxidative phosphorylation occurs in *A. vinelandii* membranes, but oxidation of hydrogen is totally absent in membranes of *R. leguminosarum*. Hydrogen oxidation in *A. vinelandii* is sensitive to increasing O_2 tensions; both respiration rate and P:O ratio decline. This result contrasts with the NADH oxidase activity in *A. vinelandii*, where, on appearance of O_2 in the medium, a decline is found in the P:O ratio, but not in ATP concentration. This decrease in P:O ratio is thus due to non-energy-linked oxidation of this substrate by O_2.

Flavodoxin hydroquinone is the electron donor for N_2ase in *A. vinelandii* and *R. leguminosarum*. NAD(P)H flavodoxin oxidoreductase is present in both organisms. This purified FAD-containing protein is able to reduce flavodoxin to both the hydroquinone and the semiquinone, but this property is insufficient for electron donation to N_2ase in the absence of energized membranes.

The O_2 stability of the *A. vinelandii* complex is achieved by formation of a tight high molecular weight ($1\text{–}2 \times 10^6$) stoichiometric complex of Av1 and Av2 with FeS II. In this complex, whose formation is promoted by Mg^{2+}, the three proteins are in the oxidized state. Reduction of the complex leads to N_2ase activity and a lowering of the molecular weight to 300,000, possibly by dissociation of this three-component complex into the usual two-component N_2ase. The protection of nitrogenase activity toward O_2 inactivation by FeS II occurs only with the intact Av1 plus Av2 complex and not with the individual component proteins.

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IV. ON THE FORMATION OF AN OXYGEN-TOLERANT THREE-COMPONENT NITROGENASE COMPLEX FROM *AZOTOBACTER VINELANDII*

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Conditions are defined in which the oxygen-labile nitrogenase components from *Azotobacter vinelandii* can be protected against oxygen inactivation by the so-called Fe/S protein II. It is demonstrated that oxygen protection can be achieved by complex formation of the three proteins. Complex formation was studied by gel chromatography. Only when the three proteins are in the oxidized state and $MgCl_2$ is present, can an oxygen-tolerant complex be isolated. Quantitative SDS/polyacrylamide gel electrophoresis of such complexes, yielded an average ratio of nitrogenase component 2/nitrogenase component 1 (Av_2/Av_1) of 2.4 ± 0.5. Protection by Fe/S protein II was correlated with the amount of [2Fe-2S] clusters present in the protein and not by the amount of protein. Measurements of the amount of iron and sulfide of Fe/S protein II showed that the iron and sulfide content of the protein was variable. The maximum values found indicate that Fe/S protein II contains two [2Fe-2S] clusters per dimer of 26 kDa. Full protection by Fe/S protein II was obtained with a ratio of Fe/S protein II/Av_1 of 1.1 ± 0.2; the Fe/S protein II containing two [2Fe-2S] clusters per dimer of 26 kDa. When Fe/S protein II contains less [2Fe-2S] clusters, more protein is necessary to obtain full protection. The three-component nitrogenase complex is also oxygen stable in the presence of MgATP or MgADP.

Analysis in the ultracentrifuge showed that the major fraction of the reconstituted complex has a sedimentation coefficient centered around 34S. A small fraction (< 30%) sediments with values centered around 11 1 S. This suggests an average mass for the oxygen-stable nitrogenase complex of 1.5 MDa. Taking into account the determined stoichiometry of the individual proteins, the molecular composition of the oxygen-stable nitrogenase complex is presumably 4 molecules of Av_1, 8 – 12 molecules of Av_2 and 4 – 6 molecules of Fe/S protein II containing two [2Fe-2S] clusters per dimer of 26 kDa.

Introductions to scientific papers on studies on the nitrogenase proteins, often start with the statement that nitrogenase can be separated into two O_2-sensitive redox proteins, or words of that kind. It is therefore noteworthy that actually so little work has been devoted towards a study of the O_2-sensitivity of these proteins. This is especially surprising since, in general, awareness has been growing among biologists that oxygen and oxygen derivatives are potentially harmful agents in all biological systems [1].

The phenomenon discovered in this laboratory, the presence in *Azotobacter vinelandii* of a Fe-S protein protecting nitrogenase from being inactivated by O_2, may provide a model for other protective mechanisms against O_2 [2,3]. In its details the system is unique as far as we know; only in *Azotobacter chroococcum* a system similar to the one we found in *A. vinelandii* has been reported [4]. A precise study of the protective mechanism in both species has not been made until now. The fact, however, that the molecular mass of the protecting Fe-S protein in *A. chroococcum* [4] is approximately half that of *A. vinelandii* [5], while both contain one [2Fe-2S] cluster [4 – 7], is intriguing.

General aspects of O_2 tolerance in nitrogen-fixing organisms have been adequately reviewed by Yates and Jones [8]; of these, especially the so-called 'switch off/on' phenomenon appears to be relevant for our results. Here we will only reiterate some of our earlier results on the *A. vinelandii* protective system [2,3]. Since Av_2 is far more sensitive to inactivation by O_2 than Av_1, in studying the inactivation of the combined proteins by O_2, one is actually studying the inactivation of Av_2. However, it appears that for the maximum protection of Av_2 by the protective protein Fe/S II, the presence of Av_1 is indispensable [2,3]. Furthermore, whereas $MgCl_2$ diminishes the protection of Av_2 against oxygen by Fe/S II [3], in the presence of Av_1 it strongly enhances the degree of protection rendered by Fe/S II [3]. The three proteins Av_1, Av_2, and Fe/S II form, in the presence of $MgCl_2$, a nitrogenase complex which is as stable as the nitrogenase complex, isolated as such from *A. vinelandii* by the procedure of Bulen and LeComte [9,10]. In titration experiments, at a fixed Av_2/Av_1 ratio of 2/1, an Fe/S II/Av_2 ratio of 1/1 was practically saturating [3]. Lastly it was shown that a very efficient electron transfer from Av_2 to Fe/S II can take place [3]. The latter finding fits our hypothesis that the above-mentioned 'switch off/on' phenomenon in whole cells can be explained by a redox-dependent complex formation of Av_1, Av_2 and Fe/S II; specifically, 'switch off' would occur by oxidation of Av_2 by Fe/S II, bringing about the formation of the oxidized, O_2-tolerant, 'switched off' complex of the three proteins. 'Switch on' can be explained by reduction of the complex followed by dissociation.

Abbreviations. The Mo-Fe and Fe proteins of *Azotobacter vinelandii* are referred to as Av_1 and Av_2 respectively; Fe/S II stands for Fe/S protein II from *A. vinelandii*; SDS, sodium dodecyl sulfate; PMS, phenazine methosulfate; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-amino}ethane sulfonic acid; PSC, protamine sulfate complex.

In this paper we will present data supporting the view that the oxidation state, the presence of $MgCl_2$ as well as the molecular ratio of the three porteins are factors in the formation of an oxygen-tolerant complex. This complex can be separated from the individual proteins by gel filtration; its molecular mass was studied in the ultracentrifuge and its composition analyzed with SDS/polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Growth conditions and protein preparation

Azotobacter vinelandii ATCC 478 was grown in a batch culture of 2500 l, harvested during the logarithmic phase and stored at −70 °C [11]. 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 as described earlier [11], 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 [9]. The so-called Fe/S protein II was purified from the fraction eluted with 80 mM NaCl from the DEAE-cellulose column used for the separation of the nitrogenase proteins. The Fe/S protein II was diluted with 5 vol. of air-saturated buffer to oxidize dithionite and the protein. Fe/S II was further purified as described by Shethna et al. [5]. The oxygen-tolerant nitrogenase complex was isolated according to Bulen and LeComte [9] up to the first $MgCl_2$ precipitation: this is the so-called protamine sulfate complex (PSC complex).

Desalting of protein samples was done by running a small volume (up to 1 ml) through a Sephadex G-25 medium column (15 × 1 cm), equilibrated with the desired buffer. Anaerobicity, if desired, was ascertained by twice running a volume of 1 ml 0.2 M $Na_2S_2O_4$ through the column, while constantly flushing the space above the gel with purified argon.

Analytical methods

Standard nitrogenase assays were run as described before, in a mixture containing 25 mM Hepes/KOH, 5.3 mM ATP, 13.3 mM $MgCl_2$, 10 mM creatine phosphate, 0.2 mg creatine kinase/ml, 1 mg bovine serum albumin/ml, final pH 7.4 [11]. Assay mixtures were made anaerobic by evacuating and filling with argon five times. Dithionite was added (20 mM, final concentration) and acetylene, purified by storage for at least 24 h above a Fieser solution, was added to a final concentration of 20%. Reactions were started by the addition of nitrogenase with gas-tight syringes.

Nitrogenase components used had throughout specific activities around 1800 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 calculation of protein concentrations the following molecular masses were adopted: Av_1, 220 kDa; Av_2, 63 kDa; Fe/S II, 26 kDa [11,12]. To estimate the amount of [2Fe-2S] clusters associated with the Fe/S II, $\varepsilon^{ox}_{344} = 16.6\ mM^{-1} \cdot cm^{-1}$ for one [2Fe-2S] cluster was used [6].

Analytical chromatography was performed on a column (30 × 1 cm) of Ultrogel AcA 34 (LKB); 2 cm of Sephadex G-25 (medium grade) was layered on top. The column was run with downward flow at a rate of 25 ml/h, maintained by a peristaltic pump. Tubing was gas-tight, except for that running through the peristaltic pump. Anaerobicity was maintained by continuously sparging elution buffers with purified argon, while at least once every 24 h a small volume of 0.2 M dithionite was run through the column. The peristaltic pump was positioned between the outlet and the monitoring spectrophotometer. The column was kept at room temperature.

Oxidation of protein samples to be run on the column, was done by first applying 0.5 ml of 20 mM PMS to the column, next 0.5 ml of eluant, and then 0.75 ml of the reduced protein sample. The protein sample overtook PMS while it was still in the Sephadex layer, so that the reaction time was minimized. Protein mixtures were oxidized by low oxygen concentrations in the reaction chamber of an oxygraph with a total volume of 1.4 ml (Rank Brothers, Bottisham, Cambridge, England). A dithionite-free protein sample (0.7 ml) was incubated under argon and oxygen was added to a final concentration of 3×10^{-3} atm (304 Pa). As soon as the dissolved oxygen concentration exceeded 3 μM, the protein mixture was transferred to a bottle flushed with argon.

Polyacrylamide gel electrophoresis was performed according to Voordouw et al. [13] using 12.5% acrylamide. For qualitative purposes, slab gels were used; for quantitative determinations, tube gels. In the latter case, calculation of the amount of protein per band was done by back reference to a calibration curve in which the extent of staining (in arbitrary units, derived from scanning the gel at 540 nm in a Gilford spectrophotometer) was plotted against the amount of protein applied.

Oxygen inactivation experiments were performed as follows. A volume of anaerobic protein solution was mixed with O_2-saturated buffer ($[O_2] = 1.16$ mM) inside a glass syringe. For easy mixing, glass beads were included. Reaction was at room temperature, in 25 mM Tes/KOH buffer (pH 7.2), 5 mM $MgCl_2$ and an O_2 concentration as indicated. The reaction was stopped by injecting the desired volume in an assay bottle, that had been flushed with argon and contained $Na_2S_2O_4$ to give a final concentration of 10 mM. Remaining activity was assayed as indicated above.

Iron was extracted from Fe/S II by adding an aliquot of the protein solution to 30% (w/v) CCl_3CO_2H giving a final concentration of 20% (w/v) CCl_3CO_2H, followed by dilution with water to 5% (w/v) CCl_3CO_2H concentration. The denaturated protein was centrifuged and the iron content of the supernatant was determined by the method of Massey [14] with bathophenantroline disulfonate (BPS) as iron chelator instead of *o*-phenathroline disulfonate. Atomic absorption was done with an Instrumentation Laboratory aa/ae spectrophotometer 357 upon the supernatant of the CCl_3CO_2H treatment.

The sulphide concentration was estimated according to Fogo and Popowski [15] as modified by Brumby et al. [16]. Treatment with Chelex-100 (Bio-Rad) to remove non-specifically bound iron, was performed batchwise. An assay bottle (7.0 ml, stoppered with a suba seal) with 0.1 g dry Chelex-100 was evacuated and filled with argon three times. 0.2 ml of buffer solution was added. After 15 min equilibration 0.2 ml of a Fe/S-II-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-100 by a syringe, with a thin needle to prevent Chelex-100 contamination. It was checked that this procedure removes extra added iron efficiently.

Circular dichroism was measured in a Roussel-Jouan dichrograph. Protein concentrations were estimated with the Lowry method and according to Sedmak and Grossberg [17] with bovine serum albumin as standard. On purified Av_1, Av_2 and Fe/S II other protein determination methods, like the biuret method [18] and the Lowry method [19], as well as dry weight measurements upon Av_2, were performed. Dry weight measurements, as well as protein determinations by the Lowry and biuret methods on purified Av_2, showed that protein estimations by the method of Sedmak and Grossberg gave an underestimation of 25%. No correction factors were necessary for protein determinations of Av_1 and Fe/S II as compared with the Lowry and biuret methods. For all protein determinations, a value for bovine serum albumin of $\varepsilon = 1.01\ mM^{-1}\ cm^{-1}$ at 279 nm was used.

Gases and biochemicals

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 and through a scrubbing solution of photochemically-reduced methylviologen.

RESULTS AND DISCUSSION

Nitrogenase can be isolated from *Azotobacter* cell-free extracts in an oxygen-tolerant form [4, 9, 10]. This so-called nitrogenase complex is not well defined. One aspect of this complex, which so far has been neglected, is that it can be isolated only in the absence of reducing agents, suggesting that only the oxidized form is O_2-tolerant and tightly associated. In Fig. 1 the effect of reduction on oxygen tolerance of the isolated complex (PSC) is shown, along with the effect of $MgCl_2$. First, when the PSC complex is made Mg^{2+}-free by passage over Sephadex G-25, it becomes extremely O_2-sensitive, to a degree comparable to that of free Av_2 (not shown, see also [20]). $Na_2S_2O_4$-reduced as well as PMS-reoxidized preparations are similarly O_2-sensitive in the absence of $MgCl_2$ (not shown). When $MgCl_2$ is added back to the PSC complex, reversal to oxygen tolerance is complete. When the PSC complex is first reduced and then $MgCl_2$ added, there is a fast initial inactivation to 60–70% residual activity, followed by a slow inactivation like that observed with the PSC complex as originally isolated. Reoxidation of the PSC complex by PMS prior to the addition $MgCl_2$ does not, however, abolish the initial inactivation, suggesting a partially irreversible inactivation of Av_2 upon reduction.

It is clear that the reactivity of the PSC complex with oxygen is strongly and reversibly affected by $MgCl_2$. The effect of the oxidation state is much less clear, due to the fact that in these experiments O_2 acts both as oxidant and as inactivator. In our view, the observed biphasicity when starting with reduced proteins ($MgCl_2$ present) must be explained by simultaneous inactivation and oxidation by O_2: oxidation leading to complex formation and concomittant oxygen tolerance (see below). The fact that most of the original activity is left after the first phase indicates that both processes, as well as complex formation, occur at similar rates. If so, this would mean that in the cell, O_2 itself may act directly as the oxidant of nitrogenase. Given the proper conditions, a stable complex may form (thereby 'switching off' nitrogenase activity) before

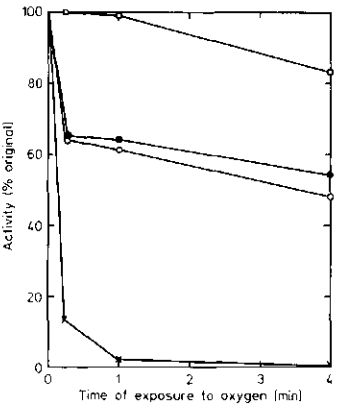

Fig. 1. *Effect of $MgCl_2$ and oxidation state on the oxygen stability of the isolated three-component nitrogenase complex from* A. vinelandii. The three-component nitrogenase complex (PSC complex) was isolated as described in Materials and Methods. Protein concentration during O_2 inactivation was 0.73 mg/ml; nitrogenase was exposed to 0.50 mM O_2 under the conditions indicated. Original activity was 210 nmol C_2H_4 formed · min^{-1} · (mg protein)$^{-1}$. (×---×) Mg-free complex; this has been used as starting material for the following treatments: (□---□) 5 mM $MgCl_2$ added; (●---●) reduced by $Na_2S_2O_4$ until no further decrease in absorbance of 430 nm was observed, after which 5 mM $MgCl_2$ added; (○---○) likewise reduced, followed by PMS until no further increase in absorbance at 430 nm was observed, after which 5 mM $MgCl_2$ added

appreciable damage occurs. Of course, the physiological relevance would be dependent on conditions, e.g. concentrations of proteins and oxygen as they occur in the cell. To what extent the isolated nitrogenase PSC complex resembles the 'switched off' state of nitrogenase *in vivo* remains to be seen.

We have reported earlier [2, 3] that it is possible to obtain a highly oxygen-tolerant nitrogenase simply by mixing dithionite-free Av_1 and Av_2 with Fe/S II in the presence of $MgCl_2$. However, in these earlier experiments, the role of redox state was not investigated properly. Also, the lack of purity of Av_1 has been a matter of concern in the past. In the experiments described here Av_1 with a specific activity of at least 2400 nmol C_2H_4 formed min^{-1} · mg Av_1^{-1} and pure, as judged by SDS/polyacrylamide gel electrophoresis, was used. It was also found that the iron content of the Fe/S II varied from preparation to preparation. As already shown by Scherings et al. [7] Fe/S II looses iron upon oxygen oxidation. During the initial purification steps dithionite is present. To maintain a high amount of iron and sulfide associated with the protein, it is important to dilute the dithionite-reduced protein solution with excess of air-saturated buffer so that dithionite and the protein are oxidized at once and keeping the number of oxidation-reduction cycles of the protein as small as possible. From a typical isolation Fe/S II was obtained with 3.4 ± 0.2 atoms iron and 3.6 ± 0.3 atoms sulfide per 26-kDa dimer. A Chelex-100 treatment on this protein indicated that the amount of non-specifically bound iron was negligible (not shown). The molecular absorbance of the oxidized and reduced protein in the visible part of the spectrum was 1.7 times the published data of Fe/S II which contained 2.0 iron and 2.2 sulfide atoms per 26-kDa dimer [6]. The shape of the visible spectrum and

EPR spectrum of Fe/S II and the temperature dependency of the intensity of the EPR signal was not different from the published data [6, 7], indicating [2Fe-2S] clusters present in the protein. The integration of a typical EPR spectrum of Fe/S II containing 3.6 atoms iron per 26 kDa indicated 1.2 unpaired electrons to be present per 26 kDa. Theoretically this number should be 1.8 unpaired electrons for this protein preparation. The reason for the discrepancy is not known but spin coupling can not be excluded. Maximum values of iron and sulfide associated with the protein found were 3.9 ± 0.2 atoms iron and 4.1 ± 0.4 atoms sulfide per 26 kDa. On a calibrated SDS/polyacrylamide gel Fe/S II behaves as a 14-kDa polypeptide. The native protein behaves on a calibrated analytical molecular sieve column as a particle of 26 kDa (see also Fig. 2). The molecular mass of the protein as determined with sedimentation equilibrium using the partial specific volume $\bar{v} = 0.74$ ml/g [6] was 24 kDa. It is therefore reasonable to suppose that native Fe/S II from *Azotobacter vinelandii* exists as a dimer of two identical subunits each with a [2Fe-2S] cluster bound. As will be shown below, the protection properties of Fe/S II depends upon the amount of [2Fe-2S] clusters. Therefore in all experiments the concentration of active protective protein was calculated by the concentration of [2Fe-2S] clusters associated with the protein taking the molecular absorbance of one [2Fe-2S] cluster of the oxidized at 344 nm as $\varepsilon^{ox}_{344} = 16.6\,mM^{-1} \cdot cm^{-1}$ [6].

The gel filtration experiments described here, were designed to prove the hypothesis that both Mg^{2+} and an oxidized state of the proteins are necessary, not only to obtain oxygen tolerance, but also to obtain a tightly associated complex. To study complex formation with gel filtration, the behaviour of the individual proteins on the gel have to be known. In Fig. 2 a calibration curve of the gel filtration column is shown. The proteins used for the calibration, as well as the nitrogenase proteins and Fe/S II, were eluted in the presence of 90 mM $MgCl_2$ to minimise specific ionic interactions. From the observed elution volumes of Av_1, Av_2 and Fe/S II, indicated by arrows, the following molecular masses of Av_1, Av_2 and Fe/S II in the presence of 90 mM $MgCl_2$ were calculated as 257, 64.6 and 25.7 kDa respectively. Of course, these values should be taken with caution: especially that of Av_1. Although according to manufacturer's specification, the separation range of the gel (LKB Ultrogel AcA 34) should be from 20 to 400 kDa, in our hands xanthine oxidase (M_r = 275000) was eluted in the void volume. The elution volumes for Av_1, Av_2 and Fe/S II were also determined under the conditions used for complex formation, i.e. low ionic strength. At low ionic strength the behaviour of the three proteins changes significantly on the molecular sieve column. In all cases the proteins move faster through the gel, indicating large conformational changes or polymerization. For all three proteins, polymerization is observed at low ionic strength. But when 5 mM $MgCl_2$ was present, Fe/S II and Av_2 seem to depolymerise partly. In the case of Av_1, 5 mM $MgCl_2$ leads to a pronounced polymerization. In fact, we use precipitation of Av_1 at 10 mM $MgCl_2$ as a routine purification step [11]. For the gel filtration experiments this meant that with 5 mM $MgCl_2$ present, a large part of the Av_1 sample stuck on top of the column; Av_1 which was eluted usually formed a broad smear, indicated by two symbols for one condition in Fig. 2. Without $MgCl_2$ added, or with Av_2 and Fe/S II present, however, such precipitation was not observed.

For our purposes, the main conclusions to be drawn from Fig. 2 are the following. Firstly, any free Fe/S II elutes fully separated from Av_1, Av_2 and any complex formed, under all conditions studied. Secondly, free Av_2 elutes fully separated from Av_1, Fe/S II, and any complex under all except the oxidizing, minus $MgCl_2$, condition. Thirdly, Av_1 in any of the conditions considered for complex formation elutes close to the void volume and is therefore not separated from any complex formed.

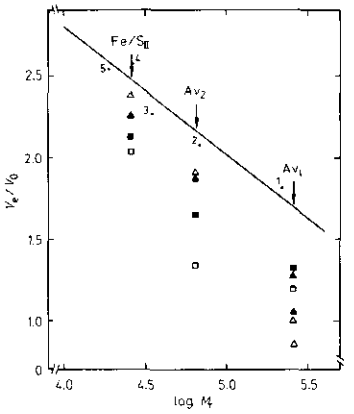

Fig. 2. *The behaviour of the nitrogenase proteins and the Fe/S protein II from* A. vinelandii *on a gel filtration column under different conditions of oxidation state and absence/presence of* $MgCl_2$. Column set-up as described in Materials and Methods. For calibration, the column was equilibrated with argon-saturated 25 mM Tes/KOH (pH 7.2), 90 mM $MgCl_2$. Void volume as determined with blue dextran was 21.8 ml. The five proteins used for calibration were: (1) catalase; (2) bovine serum albumin; (3) ovalbumin; (4) chymotrypsin A; (5) myoglobin. Arrows indicate the experimentally found log M_r values for Av_1, Av_2 and Fe/S II under calibration conditions. In the case of Av_1 and Av_2, $Na_2S_2O_4$ (2 mM) was also present in the elution buffer. The other conditions were: (△- - -△) PMS-oxidized proteins, 5 mM $MgCl_2$; (▲ · ▲) 2 mM $Na_2S_2O_4$, 5 mM $MgCl_2$; (□ · □) PMS-oxidized proteins; (■- - -■) 2 mM $Na_2S_2O_4$. In all cases, buffer was 25 mM Tes/KOH, pH 7.2

When purified Av_1, Av_2 and Fe/S II mixed in a molar ratio of 1/1.2/1, were subjected to gel filtration under the four conditions indicated, the elution patterns as shown in Fig. 3 were obtained. Peak fractions of discrete bands were collected and analyzed for the presence of each protein, as well as specific nitrogenase activity; the results are given in Table 1.

Under reducing conditions (Fig. 3A) with $MgCl_2$ present, two overlapping bands elute near the void volume. Both the first band (Table 1) and the second band (not shown) consist only of Av_1. It should be noted here, that while Av_1 *per se* in these conditions will precipitate to a large extent (see above) in mixtures of the three proteins, Av_1 does not precipitate to any observable extent. The third band, eluted at around 35 ml elution volume, consists of Av_2 with some Av_1 (not shown). Thus under these conditions Av_1 elutes around elution volumes of 22 ml, 26 ml and 35 ml. When a similar gel filtration experiment was performed by starting with a mixture of Av_1, Av_2 and Fe/S II in a molar ratio of 1/4.8/2, an extra band was eluted at an elution volume of around 40.8 ml (the position of free Av_2, arrow) while the band eluted at 35 ml increased in intensity (not shown). Therefore it is likely that the band eluted at 35 ml represents Av_1 that has been prevented from polymerizing by Av_2 or the band represents a small complex

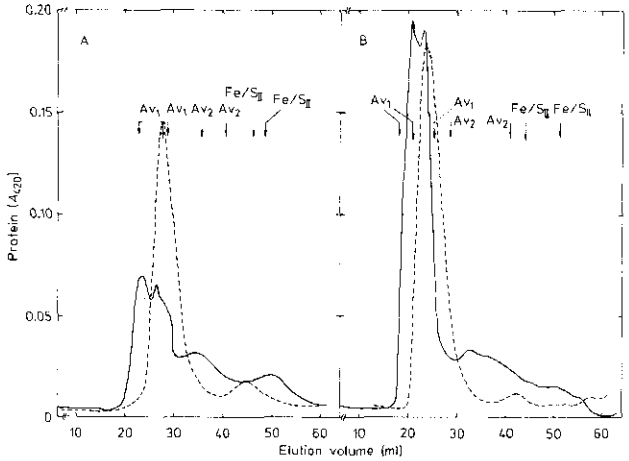

Fig. 3. *Gel filtration of the combined nitrogenase proteins and Fe/S protein II from* A. vinelandii *under different conditions of oxidation state and absence/presence of* $MgCl_2$. Column set-up and chromatography as described in Materials and Methods. Av_1, Av_2 and Fe/S II concentrations were 23 μM, 27 μM and 23 μM respectively, in a volume of 0.5 ml. The Fe/S II used contained 1.5 [2Fe-2S] clusters per dimer of 26 kDa, and the concentration of Fe/S II is expressed as the concentration of the [2Fe-2S] clusters present. Elution conditions were: (————) 5 mM $MgCl_2$; (– – – – –) no $MgCl_2$. Buffer was 25 mM Tes/KOH, pH 7.2. In A, 2 mM $Na_2S_2O_4$ was present in the eluant. In B, protein mixture was oxidized by PMS as described in Materials and Methods

Table 1. *Presence of Av_1, Av_2 and Fe/S II in high-molecular-mass fractions obtained by gel filtration of mixtures of the three proteins in the presence and absence of $MgCl_2$ and $Na_2S_2O_4$ and after oxidation with phenazine methosulfate*

Chromatography and conditions as described in Fig. 3. Fractions obtained between elution volume 20 – 30 ml (see text) were analyzed by SDS/polyacrylamide gel electrophoresis on slab gels. Per sample 5 – 10 μg protein was applied; 0.1 μg per band was well within detection range. + = present, – = absent, ± = present in amounts close to the detection limit (which means less than 2% of total protein applied). Nitrogenase activity is expressed as nmol C_2H_4 produced · min^{-1} · (mg total protein)$^{-1}$

Conditions	Av_1	Av_2	Fe/S II	Nitrogenase activity
				nmol min^{-1} mg^{-1}
Reducing, plus Mg^{2+}	+	–	–	0
Reducing	+	±	–	10
Oxidizing	+	+	±	0
Oxidizing, plus Mg^{2+}	+	+	+	250

between Av_1 and Av_2. Finally the fourth band is Fe/S II, being eluted at the elution volume of free Fe/S II.

Under reducing conditions without $MgCl_2$, it is difficult to draw any conclusions about possible complex formation of Av_1 and Av_2 from the elution patterns shown, since the elution volumes of the free Av_1 and Av_2 proteins are rather close to each other. In any case, up to an elution volume of 29 ml, only a slight amount of Av_2 and no Fe/S II at all could be detected in the eluate (Table 1), indicating no strong interaction between Av_1, Av_2 and Fe/S II.

Under oxidizing conditions (Fig. 3B) without $MgCl_2$ present, the elution pattern is similar to that under reducing conditions without $MgCl_2$; however, in the fraction collected between 24 ml and 28 ml, both Av_1 and Av_2 were present and also, notably, a slight amount of Fe/S II (Table 1). The presence of Av_2 with Av_1 is not surprising since the elution volumes of the free proteins are close; but the presence of Fe/S II and the shift of Av_1 to smaller elution volumes indicates, in our opinion, some complex formation. Notably, when PMS oxidation was omitted, the first fraction reached its peak at 26 ml (24.5 ml after oxidation and more free Fe/S II was eluted, not shown).

Under oxidizing conditions with $MgCl_2$ present, again a double band elutes near the void volume, which in this case consists of all three proteins (Table 1). The specific nitrogenase activity of this fraction as compared with that of the starting mixture (approximately 350 nmol · min^{-1} · mg^{-1}) indicates that this fraction is reasonably O_2-tolerant. One should in any case consider the specific activities given in Table 1 as reflecting to some degree the oxygen tolerance, because it is impossible in this kind of gel filtration experiment to exclude oxygen completely. When the column was run without dithionite, any free Av_2 collected after passing the peristaltic pump was inactive. Another feature of this elution pattern is a faint band around 30 – 40 ml elution volume in which both Av_1 and Av_2 are found (not shown in Table 1). This indicates that either free but not polymerized Av_1 or a small Av_1-Av_2 complex is present under these conditions and not all Av_1 and Av_2 are present in the high-molecular-mass fractions. This might be caused by the fact that the ratio of components is not optimal for full complex formation. When the initial ratio Av_1/Av_2/Fe/S II changed to 1/1.2/2 instead of 1/1.2/1 as in Fig. 3B, the band eluted between 30 – 40 ml disappeared. This indicates that under the conditions of almost equal amounts of Av_1 and Av_2, excess of Fe/S II is necessary

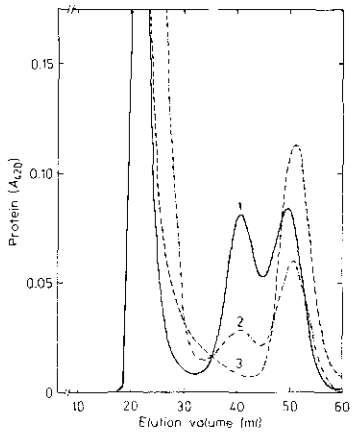

Fig. 4. *Gel filtration of the combined nitrogenase proteins and Fe/S protein II from* A. vinelandii *under oxidizing conditions, with 5 mM $MgCl_2$ present at different molar ratios.* Column set-up, chromatography and oxidation of the protein mixture as described in Materials and Methods. Protein concentrations in the mixtures, before applying them to the column, were: curve 1 (——) 22 μM Av_1, 121 μM Av_2, 124 μM Fe/S II; curve 2 (· · ·) 32 μM Av_1, 88 μM Av_2, 90 μM Fe/S II; curve 3 (– –) 22 μM Av_1, 26 μM Av_2, 124 μM Fe/S II. The Fe/S II used contained 1.5 [2Fe-2S] clusters per dimer of 26 kDa and the concentration of Fe/S II is expressed as the concentration of [2Fe-2S] clusters present. Total volume applied was, in experiments 1 and 3, 0.53 ml, and in experiment 2, 0.73 ml. The fractions that were eluted at around 21.6 ml (22.7 ml in the case of experiment 2) reached peak absorbance at 430 nm of (1) 0.295, (2) 0.485, (3) 0.245

for the formation of a high-molecular-mass complex which contains all the Av_1 and Av_2.

So, it is evident that, of the four conditions applied, only when 5 mM $MgCl_2$ is present and the proteins are oxidized, is a stable three-component complex formed. To form this complex in the way described above, oxidation by PMS is essential. When PMS oxidation was omitted, or O_2 (i.e. O_2-saturated buffer) was used as an oxidant, we did not observe co-elution of the three proteins or nitrogenase activity in any fraction eluted from the column.

In Table 1, a specific activity of 250 nmol · min^{-1} mg^{-1} for the nitrogenase-complex-containing fraction indicates an Av_2/Av_1 ratio of less than 1 (assuming all Av_2 is active). This raises the question of whether indeed a definite stoichiometry does exist for the three-component nitrogenase complex. In solving this problem one is somewhat handicapped by the fact that Av_1 polymerizes under the conditions of complex formation and therefore molecular sieve columns may not separate polymerized Av_1 from a higher-order three-component complex. In this respect, more conclusive results about a possible maximum stoichiometry of the three proteins of the complex can be obtained by gel filtration of mixtures of proteins with an excess of Av_2 and Fe/S II to saturate binding sites for Av_2 and Fe/S II present on Av_1 and to minimize the concentration of free (polymerized) Av_1.

Fig. 4 shows elution patterns of gel chromatography of mixtures of Av_1, Av_2 and Fe/S II in three different molar ratios under oxidizing conditions in the presence of $MgCl_2$. With fivefold excess of Av_2 and Fe/S II over Av_1, about equal amounts of free Av_2 and free Fe/S II are recovered in the eluate, assuming similar absorption coefficients (G. Scherings, unpublished results). When the concentration of Av_1 is increased and the excess of Av_2 and Fe/S II is lowered to 2.8, the amount of both free Av_2 and free Fe/S II recovered diminishes, but free Av_2 and Fe/S II are still present. This indicates that a threefold excess of Av_2 and Fe/S II over Av_1 is enough to saturate Av_1 with Av_2 and Fe/S II. When the concentration of Av_2 is lowered to equal that of Av_1 (line 3) practically no free Av_2 is recovered but considerably more Fe/S II than in the case of excess Av_2 (line 1). This indicates to us that stoichiometric amounts of Av_2 are necessary for binding of Fe/S II to the complex. The tailing of protein after the main peak in experiment 3 indicates also that complex formation is not complete or that the complex formed is not as tight as in experiment 1.

It should be possible to calculate from these elution patterns the ratio Av_1/Av_2/Fe/S II in the high-molecular-mass fractions. It appears that approximately up to 2 mol Av_2/mol Av_1 can be incorporated in the high-molecular-mass fraction (line 2). Similarly (from elution patterns not shown here) it appears that this value for Fe/S II varies between 1 and 2 on the basis of the protein concentration. On the basis of [2Fe-2S] clusters, this value approximates to two moles of Fe/S II monomer/mole Av_1. However, it is preferable to estimate more directly the composition of the high-molecular-mass fractions isolated by gel chromatography. Results are given in Table 2. Due to the fact that Fe/S II exhibits a strong circular dichroism around 420 nm, an independent measurement of the concentration of the [2Fe-2S] clusters in Fe/S II in these high-molecular-mass fractions can be made. By measuring the intensity of the CD band around 420 nm, the same results were obtained as with quantitative SDS/polyacrylamide gel electrophoresis (not shown). The results presented in Table 2 confirm those of Fig. 4. They indicate that a maximal amount of Av_2 and Fe/S II can be bound to Av_1 (compare experiments 1–3). This maximal value is around 2.5 molecules Av_2 and 2.4 molecules of Fe/S II monomer with one [2Fe-2S] cluster bound and does not depend on the ratios in the starting mixture. When the ratio of Av_2 to Av_1 was lowered below 2, less Fe/S II was also found in the high-molecular-mass complex (experiment 4). This indicates to us that Av_2 is necessary for the binding of Fe/S II to the complex. When the Fe/S II concentration is lowered in the starting mixture, somewhat less Av_2 was found in the high-molecular-mass complex and the complex was enriched with Fe/S II (experiment 5). This is possible because considerable amounts of free, not polymerized, Av_1 and Av_2 were eluted between 30–40 ml elution volume (see also Fig. 3B). This indicates that with a relative excess of Av_1 and Av_2 over Fe/S II, a Av_1/Av_2/Fe/S II complex around 1/2/2 is formed prevalently and the excess of Av_2 prevents Av_1 from polymerizing, perhaps by the formation of a smaller-molecular-mass complex.

From six chromatographic runs with a twofold or more excess of Av_2 and Fe/S II over Av_1, average molar ratios of the three components in the complex are obtained: $Av_2/Av_1 = 2.4 \pm 0.5$ and Fe/S II monors with one [2Fe-2S] cluster bound/$Av_1 = 2.2 \pm 0.4$.

As has already been stated, the specific activity of the high-molecular-mass fractions reflects some degree of oxygen stability. When these fractions were exposed to 250 μM O_2 no significant loss of activity was observed after 2 min of exposure to oxygen, whereas free Av_2 under identical conditions was completely inactivated. Free Av_1 is not inactivated

Table 2. *The presence of Av_1, Av_2 and Fe/S protein II and the specific nitrogenase activity of high-molecular-mass fractions obtained after gel filtration of phenazine-methosulphate-oxidized mixtures of Av_1, Av_2 and Fe/S protein II*

Protein mixtures of different component ratios, as indicated, were oxidized by phenazine methosulfate and eluted on a molecular sieve column in 25 mM Tes/KOH, 5 mM $MgCl_2$ final pH 7.2 sparged with argon. The Av_1 concentration in the starting mixture applied to the column in a total volume of 0.73 ml was 16.4 μM. Column specifications and chromatography are as described in Materials and Methods. High-molecular-mass fractions were peak fractions of the column eluate at approximately 20–24 ml elution volume. Ratios were determined by quantitative SDS/polyacrylamide gel electrophoresis as described in Materials and Methods. Fe/S II used contained 1.5 [2Fe-2S] clusters per dimer of 26 kDa. Fe/S II concentration is expressed as [2Fe-2S] cluster concentration. Nitrogenase activity is expressed as nmol C_2H_4 formed · min^{-1} · (mg total) $protein)^{-1}$

Expt	Starting mixture		High-molecular-mass fraction	
	ratio Av_1/Av_2/Fe/S II	nitrogenase activity	ratio Av_1/Av_2/Fe/S II	nitrogenase activity
		nmol min^{-1} mg^{-1}		nmol min^{-1} mg^{-1}
1.	1/5.4/5.5	631	1/2.0/2.4	330
2.	1/2.3/5.5	460	1/2.7/2.1	362
3.	1/2.7/2.7	462	1/2.9/2.0	413
4.	1/1.2/5.5	206	1/1.2/1.1	171
5.	1/2.6/1.4	563	1/1.9/2.1	403

Table 3. *Inactivation by O_2 of phenazine-methosulphate-oxidized or O_2-oxidized mixtures of Av_1, Av_2 and Fe/S protein II*

The concentration of Fe/S II is expressed as the concentration of [2Fe-2S] clusters present. The protein used contained 1.7 [2Fe-2S] clusters per dimer of 26 kDa. The proteins were incubated for 3 min with 10 mM phenazine methosulphate (PMS) in different ratios with $[Av_1] = 10$ μM, in 25 mM Tes/KOH, 5 mM $MgCl_2$, pH 7.2. The protein mixture was chromatographed on a Sephadex G-25 medium column equilibrated with argon-flushed 25 mM Tes/KOH, 5 mM $MgCl_2$ (pH 7.2) and exposed to 0.25 mM O_2 as described in Materials and Methods. In the case of oxidation of the protein mixture with low concentrations of oxygen, protein was made dithionite-free and oxidized by low concentrations of O_2 as described in Materials and Methods. After these treatments the protein mixture was exposed to 0.25 mM O_2 with the additions indicated and as described in Materials and Methods. Nitrogenase activity is expressed as nmol C_2H_4 formed · min^{-1} · (mg total protein)$^{-1}$

Expt	Ratios of Av_1, Av_2 and Fe/S II in the incubation mixture	Oxidation method	Nitrogenase activity	
			before O_2 exposure	2 min after O_2 exposure
			nmol min^{-1} mg^{-1}	
1.	1/2.4/2.9	–	460	115
2.	1/2.4/2.9	PMS	460	435
3.	1/2.4/2.9 plus 90 mM $MgCl_2$	PMS	460	230
4.	1/2.4/2.0	O_2	490	434
5.	1/2.2/2.9	PMS	580	388
6.	1/7.2/8.7	PMS	560	392
7.	1/2.4/11.6	PMS	450	225
8.	1/2.4/0.9	–	531	71
9.	1/2.4/0.9	O_2	302	250
10.	1/2.5/2.5 + 2 mM MgATP	PMS	436	436
11.	1/2.5/2.5 + 2 mM MgADP	PMS	436	412

after a 2-min exposure to 250 μM O_2 (not shown). The influence of the molar ratio of the proteins on the formation of an oxygen-stable preparation was examined more closely. The results are presented in Table 3. It can be seen that Av_1/Av_2/Fe/S II mixtures just above 1/2/2 after being oxidized, anaerobic with phenazine methosulfate or with low concentrations of O_2 in the presence of Mg^{2+}, are oxygen-stable (experiments 1, 2 and 4). When higher concentrations of Av_2 are present with or without higher concentrations Fe/S II, nitrogenase activity is partly inactivated by the oxygen exposure (experiments 5 and 6). The resulting specific activity around 400 nmol · min^{-1} · mg^{-1} indicates that the ratio of Av_1/active Av_2 is about 1/2.4. Fe/S II present in excess of Av_2 during oxygen inactivation destabilizes complex formation, indicated by an increased oxygen lability (experiment 7). This was already observed earlier [2]. When the concentration of Fe/S II is significantly below 2, in the presence of optimal Av_2 concentrations, nitrogenase activity is partly protected (experiments 8 and 9). Also a higher ionic strength decreases the oxygen stability, probably by dissociating the complex (experiment 3). When the anaerobic oxidation with PMS was omitted only a small portion of the initial activity (< 30%) of Av_1/Av_2/Fe/S II complexes was left after a 2-min exposure to 250 μM oxygen (experiments 1 and 8). The remaining activity was relatively oxygen-stable. When the three-component mixture was oxidized with low concentrations of O_2, an oxygen-stable complex was obtained without loosing considerable activity (experiment 4). These results indicate that the formation of an oxygen-stable nitrogenase complex by oxidation of the reduced proteins by oxygen can only be successful if nitrogenase is oxidized by low concentrations of oxygen. Also the presence of MgATP or MgADP (experiments 10 and 11) does not influence the stability of the nitrogenase activity. Such situations may occur *in vivo*, indicating that the described phenomenon might have physiological relevance.

It is obvious from the data presented in Table 2 and 3 that the average ratios of Av_1/Av_2/Fe/S II in the oxygen-tolerant complex are around 1/2.4/2.2. Indeed, an earlier titration of a mixture of Av_1 and Av_2 in a molar ratio of 1/2 with Fe/S II containing only one [2Fe-2S] cluster per 26 kDa showed that maximal oxygen tolerance was obtained at a ratio of Av_1/Av_2/Fe/S II of 1/2/2 [3]. In the experiments described here Fe/S II with 1.7 [2Fe-2S] clusters per 26 kDa was used. In both cases full protection was obtained when two [2Fe-2S] clusters were present, indicating that it is not the protein concentration which is important but rather the concentration of protein with [2Fe-2S] clusters bound. If the Fe/S II monomer is the protective species, the protective system of *A. vinelandii* resembles that of *Azotobacter chroococcum* more closely [4]. In *A. chroococcum* the protective protein is a monomer of 14 kDa containing one [2Fe-2S] cluster. But it is also possible that the Fe/S II dimer is the protective species and that in the case of *A. chroococcum* the protective protein is bound as a dimer in the oxygen-stable nitrogenase complex.

The ratio Av_1/Av_2/Fe/S II of 1/2.4/2.2 is probably a maximal ratio with respect to Av_2 and Fe/S II, since it was observed that also ratios of Av_1/Av_2/Fe/S II of 1/1.2/1 form

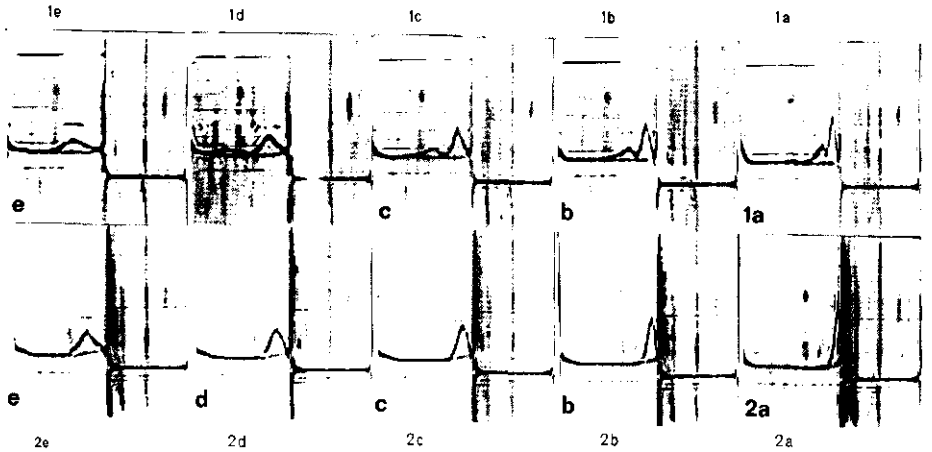

Fig. 5. *Ultracentrifuge Schlieren patterns of high-molecular-mass complexes of the nitrogenase proteins and the Fe/S protein II from* A. vinelandii *showing the effect of reduction.* The protein mixture used was obtained from a mixture of 46 μM Av_1, 92 μM Av_2 and 92 μM Fe/S II. Fe/S II used contained 1.5 [2 Fe-2S] clusters per dimer of 26 kDa and the concentration of Fe/S II is expressed as the concentration of [2 Fe-2S] clusters present. The protein mixture was oxidized with PMS and eluted on the AcA 34 gel-filtration column as described in Materials and Methods. High-molecular-mass fractions (22 – 26 ml eluate) were used for analysis in the ultracentrifuge after dilution to 1.75 mg protein^{-1} under the conditions indicated. The photographs were taken at the time indicated after reaching 30000 rev./min in an MSE analytical ultracentrifuge. Sedimentation at 20 °C was from right to left. Conditions: gas phase, argon; protein concentration, 1.75 mg/ml; bar angle, 50°; temperature, 20 °C. Experiment 1: 25 mM Tes/KOH, pH 7.2, 5 mM $MgCl_2$; at times (1a) 30 s; (1b) 100 s; (1c) 200 s; (1d) 700 s; (1e) 1400 s. Experiment 2: 25 mM Tes/KOH, pH 7.2, 5 mM $MgCl_2$, 30 mM $Na_2S_2O_4$; at times (2a) 245 s; (2b) 515 s; (2c) 915 s; (2d) 1245 s; (2e) 1830 s

an oxygen-stable complex (see Fig. 3 and Table 1). However, it cannot be excluded that in these preparations a tight Av_1/Av_2/Fe/S II complex of 1/2/2 is formed and equal amounts of free polymerized Av_1 are present.

To obtain more insight in the molecular composition of the oxygen-stable complex we studied the complex in the ultracentrifuge. From the elution pattern of the oxygen-stable complex on a Bio-Gel A-1.5 m molecular sieve column (fractionation range for globular biomolecules 10 – 1500 kDa), it was clear that the apparent molecular mass of the oxygen-stable complex was higher than 1 MDa. In the presence of MgADP, the nitrogenase dissociates and elutes as a broad peak (not shown).

The different behaviour of the nitrogenase complex in the presence of MgADP was observed earlier. Veeger et al. [3] showed that the nitrogenase activity in a crude extract of *A. vinelandii*, in the presence of Mg^{2+}, behaves as a complex of 1.3 MDa on an analytical Sepharose 4B column and as a particle of 0.4 MDa in the presence of MgADP [3].

We analyzed the high-molecular-mass fractions obtained from an AcA 34 molecular sieve column in the ultracentrifuge. In Fig. 5 the Schlieren patterns of isolated complexes as they appear during a sedimentation run are shown. It can be seen that a small portion of the protein solution sediments fast. This fraction varies between 0 and 30% of total protein. For the fast-sedimenting species, a sedimentation coefficient of 111 S was calculated. The major portion of the protein solution sediments with an average value of 34 S. Both species dissociate into smaller species when either the protein solution was reduced by the addition of 30 mM $Na_2S_2O_4$ or by an increase of the ionic strength by the addition of 400 mM NaCl. In the two cases the fastest sedimenting protein component sediments with a value of 11.2 S and 12.5 S respectively. The value for Av_1 under the same conditions is 10.3 S. This indicates that reduction or a high ionic strength, dissociates the nitrogenase complex to smaller complexes but not completely into the free components. The sedimentation values we obtained agree well with the values observed by Thorneley et al. [21] for the nitrogenase proteins from *A. chroococcum* and *Klebsiella pneumoniae*.

From our experiments, it is clear that the reconstituted oxygen-stable nitrogenase complex consists of multiples with the stoichiometric ratio of Av_1/Av_2/Fe/S II = 1/2.4 ± 0.5/2.2 ± 0.4. A sedimentation of 34 S is indicative of a molecular mass around 1.5 MDa, assuming the particle is globular [22]. When the complex consists of four molecules of Av_1 the molecular mass of the oxygen-stable nitrogenase complex is 1.5 – 1.9 MDa. In the presence of 2 mM MgADP, the sedimentation behaviour of O_2-stable nitrogenase becomes complex. The complex dissociates into different species with a maximum sedimentation of 26 S (not shown). As shown in Table 3 (experiment 11), these particles are still oxygen-stable.

To what extent the reconstituted three-component nitrogenase complex resembles the physiological 'switched off' nitrogenase remains to be seen. Important factors are the ratio of the three proteins in the cell, the Mg^{2+} concentration and the possibilities for oxidizing the three proteins inside the cell without damaging the Fe-protein. Our results indicate that at low free-oxygen concentrations it is possible to obtain a 'swith off' nitrogenase complex with preservation of the Av_2 activity, indicating that the described phenomena are not, a priori, unphysiological.

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V. GENERAL DISCUSSION AND CONCLUSION

From the preceding chapters, the following statement emerges:

Nitrogen fixation in Azotobacter vinelandii has two features which, in combination, set it apart from nitrogen fixation in most other species: (1) electron donation via a flavoprotein with an unusually low redox potential ($E_{m,7}$ = -495 mV) and (2) operating optimally at relatively high external oxygen concentrations. Apparently, A.vinelandii manages to reconcile the contradictory demands of strongly reductive (nitrogenase activity) and strongly oxidative (energy conversion through respiration) processes in one and the same cell exceptionally well.

This statement will be discussed below. As main topics can be discerned, subsequently: flavodoxin as the physiological reductant for nitrogenase; the mechanism of switch-off; and the formation *in vitro* of an oxygen-tolerant complex.

Flavodoxin: the physiological reductant for nitrogenase?

It should be made clear at the outset that the proposal that flavodoxin is the physiological electron donor for nitrogenase in *Azotobacter* (chapter II), though now commonly accepted as fact (22) does need additional proof, in line with that obtained in *K.pneumoniae* (3,20).

Arguments *pro* and *contra* may be summarized as follows.

(a) Benemann *et al.* (2), using glucose-6-P, isocitrate, or malate plus NADPH, found in cell-free extracts a low nitrogenase activity. By DEAE-cellulose treatment of the cell-free extract they found that this activity was dependent on both ferredoxin and flavodoxin, as well as on two unidentified factors, one of which could be replaced by spinach ferredoxin: NADP oxidoreductase. On the basis of these results, Yoch (33) proposed a linear pathway for electrons, from the carbon substrate to nitrogenase: via NADP to ferredoxin to flavodoxin to nitrogenase. Flavodoxin was proposed to be the ultimate reductant of nitrogenase, since it was found in the cell in nearly equimolar concentration with the nitrogenase, whereas ferredoxin I was found at about one-tenth of this concentration. Unfortunately, these endogenous nitrogenase activities were so low that their physiological significance must be regarded questionable. In

fact, nitrogenase activity in *Azotobacter* cell-free extracts with endogenous factors only has been reported by two other labs including the very early report of Bach *et al.* (1,31).

(b) Van Lin and Bothe (29), using chloroplasts to generate sufficient reducing power, found that both flavodoxin and ferredoxin I could serve independently as e^--carriers between chloroplasts and nitrogenase. Using this system, ferredoxin I at 80 μM could stimulate nitrogenase to approximately 160% of dithionite activity; flavodoxin to less than 40%. However, it should be clear that the use of chloroplasts may lead to rather 'murky' results: the effect of ferredoxin may have been mediated by substances leaking from the chloroplasts. Moreover, an unpurified nitrogenase preparation was used (obtained by centrifugation). Also, the purity of the flavodoxin used was not stated; the final purification step, however, consisted in elution from a DEAE-cellulose column with a linear KCl-gradient, whereas in my hands, usually a reversed gradient of ammonium sulphate was necessary to obtain purity.

(c) Yates (32), adroitly circumventing the complexities of chloroplasts, used dithionite-reduced flavodoxin in substrate amounts. He showed that purified nitrogenase components could reoxidize the reduced flavodoxin, at quite a reasonable rate. Still, his results were rendered somewhat inconclusive by the consideration that traces of SO_2^- might have served as an intermediate between flavodoxin and nitrogenase.

(d) Scherings *et al.* (this thesis, Ch. II) took advantage of a newly developed method of flavin-photoreduction. We used a 5-deazaflavin which appears to be a superior photoreductant in the presence of a nitrogen-containing donor, such as EDTA or tricine. Both flavodoxin and ferredoxin could be reduced this way; to a larger extent than was possible using dithionite. In assays with deazaflavin, tricine and continuous illumination, flavodoxin turned out to be a far more efficient e^--carrier for nitrogenase than ferredoxin. Furthermore, it was shown that substrate amounts of photoreduced flavodoxin could be oxidized by nitrogenase, at higher rates than dithionite.

Upon reviewing this evidence, there can be little doubt that, choosing between flavodoxin and ferredoxin I, flavodoxin is the more likely candidate to serve as ultimate reductant for nitrogenase *in vivo*, in *Azotobacter*. Whether it is the one and only reductant for nitrogenase, however, is still open to experiment. Bothe *et al.* (4) expressed serious doubts concerning the candidacy of flavodoxin, mainly on the grounds that it would be 'unusual' among nitrogen

fixers. It is easy to respond to this now: firstly, exceptions *do* exist, and secondly, definite proof has been obtained by genetic experiments that in *K.pneumoniae* a gene coding for flavodoxin, not ferredoxin, is part of the *nif*-gene cluster (3,20).

It appears that in *Azotobacter*, too, the last word has to come from genetic experiments. As already mentioned in Introduction (Ch. I), Klugkist *et al.* (19) have obtained evidence that flavodoxin synthesis in *A.vinelandii* may be at least partially *nif*-specific. True, flavodoxin was also detectable in NH_4^+-repressed cells, but in lower amounts than in N_2-grown cells. Since the flavodoxin present in NH_4^+-grown cells shows a slightly different behaviour on two-dimensional SDS-polyacrylamide gels from the flavodoxin present in N_2 fixing cells (Klugkist, pers. commun.), it may be possible that different species of flavodoxin are involved in the two modes of growth. Kennedy and Robson (18) have constructed a plasmid containing *nif A* from *K.pneumoniae* and a kanamycin resistance gene. *A.vinelandii* regulatory NIF^--mutants could be transformed by this plasmid; transformed cells showed constitutive *nif*-expression. Such plasmids, together with appropriate mutants, may be very useful tools, also to resolve the particular problem considered here.

As already discussed in the Introduction of this thesis (I.2.2), a main problem with proposing flavodoxin as the reductant for nitrogenase is the fact that (so far) it has not been possible to demonstrate significant reduction of flavodoxin (to the hydroquinone state) in cell-free extracts. However, some evidence has been presented that an NADH-flavodoxin oxidoreductase is indeed present (5). Since in cell-free systems with NADH as an electron donor only the quinone → hydroquinone $2e^-$-reduction step ($E_{m,7}$ = -270 mV) would be practically feasible, whereas in nitrogenase reduction the hydroquinone/semiquinone couple functions (chapter I), an additional enzyme catalyzing the oxidation of semiquinone to quinone would be needed. For a more extensive discussion of the possible role of flavodoxin in nitrogen fixation and the problems involved, see Haaker (13). Despite these uncertainties, in my opinion flavodoxin must be considered the most likely candidate to serve as ultimate e^--carrier to nitrogenase. Moreover, it will be discussed below that the organism's choice for flavodoxin instead of ferredoxin would make perfect physiological sense.

Nitrogenase reaction with flavodoxin as e^--donor

Most enzymological studies so far have utilized the artificial e^--donor dithionite; however as emphasized in the Introduction (Ch. I.2.3), results obtained

that way should not be extrapolated to the *in vivo* situation without further ado. Similarly, to stay as close to physiology as possible, a semi-purified nitrogenase preparation (the so-called Bulen-LeComte complex) was used instead of purified components (Av_1 + Av_2). This complex contains besides Av_1 and Av_2 a number of 'contaminating' proteins (see below).

It so turned out that indeed this 'physiological' reaction exhibited some striking differences as compared with the standard nitrogenase reaction measured with Av_1 + Av_2 and dithionite as e^--donor. The reaction may be characterized as follows.

(a) *A.vinelandii* flavodoxin during steady-state electron transport to nitrogenase shuttles between the hydroquinone ($FldH_2^-$) and semiquinone ($FldH_2^{\cdot}$) forms; the oxidized form has no part in the reaction.
In itself this is a noteworthy finding in view of the assertations by Braaksma *et al.* (6) that Av_2 may contain two [4Fe-4S] clusters and thus accomodate 2 electrons. However it is likely that Av_2 in the Bulen-LeComte complex has lost some Fe, considering its low specific activity (usually less than 200 nmoles C_2H_4 formed.min^{-1}.mg total $protein^{-1}$.

(b) The maximum rate of electron transport exceeds that when using dithionite as an e^--donor, and is dependent on the initial ratio $FldH_2^-/FldH_2^{\cdot}$ ('starting potential').
The fact that nitrogenase activities using physiological e^--donors may exceed those obtained with dithionite has also been observed by Hageman and Burris (14) and Van Lin and Bothe (29). Since the rate-limiting step in nitrogenase activity is commonly assumed to be dissociation of the (Av_{2ox}.Av_1) complex (step 4 in Scheme 1, Ch. I.1.1) (16,28), two explanations may be offered: (a) flavodoxin and/or ferredoxin may render the (Av_{2ox}.Av_1) complex less stable, or (b) they may be able to reduce Av_{2ox} while it is still complexed to Av_1, -something which dithionite apparently is not able to do.
The effect of 'starting potential' on the rate of e^--transfer in steady-state is a phenomenon which at present can only be speculated about. It would appear most likely that the effect resides in the 'true' nitrogenase, Av_1. In the scheme of Thorneley and Lowe (28), Av_1 can assume a range of redox levels, with electrons located in P- or M-clusters or involved in bond formation to different reducible substrates (cf. Introduction, I.1.1). According to this scheme, different substrates require different redox levels (or: a different

number of $1e^-$-transfers between Av_2 and Av_1). This way, reduction of H^+ by Av_1 would require at least 2 electron transfers from Av_2 to Av_1; reduction of N_2 would require at least 4 transfers (starting from the dithionite-reduced state). However, there is no obvious reason why also the higher-reduced states might not contribute to H^+-reduction in the absence of N_2. Perhaps a low 'starting potential' of flavodoxin might induce and maintain a more reduced state of Av_1 than would be the case in the usual dithionite-supported H^+-reduction by nitrogenase, and thereby enhance the rate of e^--transport. Admittedly, this would violate the common feeling that the rate of e^--flow through nitrogenase is constant, independent from the type of substrate used (at saturating e^--donor and MgATP concentrations, of course). The 'anomalous' behaviour of nitrogenase when using flavodoxin as an e^--donor instead of dithionite in any case emphasizes the point made earlier: that key enzymological findings should be re-checked with the physiological e^--donor, before extrapolations to the *in vivo* situation are made.

(c) Oxidation of substrate quantities $FldH_2^-$ by H^+ via the Bulen-LeComte nitrogenase complex, shows three characteristic phases in time: (1) an initial lagphase, (2) a constant rate at declining $FldH_2^-/FldH_2^\cdot$ ratio till that ratio reaches a value corresponding to a calculated redox potential of approximately -475 mV, and (3) above -475 mV a very steep decrease in oxidation rate, down to zero at approximately -460 mV.

In Ch. II, discussing Fig. 5, the intial lagphase has been ascribed to the presence of the major 'contaminating' protein in the Bulen-LeComte complex, the so-called Fe/S protein II. Other workers have observed lagphases in H_2-evolution by purified Av_1 + Av_2, using dithionite as an e^--donor (15), and ascribed these to a 'filling up' of Av_1 with electrons before steady-state substrate reduction was attained. The lagphase discussed in Ch. II, of course, was observed at the *input* side of electrons in nitrogenase, and might reflect dissociation of a tight complex of proteins rather than any particular events in one of the components of such a complex. It is well-known that dithionite-free Ac_1 + Ac_2 will form a tight 1:1 complex which can be observed in the ultracentrifuge (27) and that the presence of dithionite will weaken the interaction between the two proteins. Thus it might be expected that deliberate oxidation of the two proteins would render the complex even more stable. This, however, is speculation. Similarly, the results shown in Ch. II, Fig. 5, ascribing the lagphase only to the presence of Fe/S II, are inconclusive mainly because the redox state of the proteins has not been controlled. Thus,

the induction of a lagphase that was observed when Fe/S II was added to a mixture of dithionite-free (reduced?) Av_1 + Av_2 might perhaps simply be explained by a partial oxidation of Av_1 and/or Av_2 by the oxidized Fe/S II. I still feel, however, that Fe/S II is responsible for the larger part of the lagphase, simply because the three-component complex is more stable than the two-component (Av_1 + Av_2) complex. This will be discussed below. In any case, Fig. 5 unequivocally shows that pre-reduction of a mixture of the three proteins: Av_1, Av_2, and Fe/S II in some undefined but certainly not fully reduced state, leads to abolishment of the lagphase. This observation provoked the postulate that the phenomenon of 'switch-off' that can be observed in whole cells (I.2.3 *c)* should at the molecular level be explained by a *redox-dependent complex formation* of the three major proteins from the crude nitrogenase complex: Av_1, Av_2 and Fe/S II. The redox state of the couple $FldH_2^-/FldH_2^{\cdot}$ might serve as 'trigger' for complex association/dissociation. This postulate has been included in a physiological scenario which describes molecular events during switch-off, below.

Scenario for oxygen protection of nitrogenase

The following sequence of events may be envisaged. (a) The oxygen supply approaches the limit of a given respiration capacity in an *A.vinelandii* cell and increases. (b) Available reducing equivalents are being abducted to O_2 as much as possible; the respiratory chain gets more oxidized; in short, the available 'reducing power' declines to a level lower than would be needed to be able to reduce flavodoxin semiquinone. (c) The redox potential of the couple $FldH_2^-/FldH_2^{\cdot}$ approaches -475 mV and rises. (d) O_2 gets inside the cell. (e) $FldH_2^-$ is being oxidized very quickly now by O_2. The electron flow to nitrogenase thus has been cut off. (f) Av_1 and Av_2 are being oxidized by N_2 or H^+ (MgATP is at a high level in these conditions!) and form an inactive, oxygen-tolerant complex with Fe/S II.

A few comments concerning the above scenario may be made, especially with regard to the last part.

(1) Flavodoxin semiquinone might play a specific role in the formation of the oxygen-tolerant complex, for the following reasons. Comparison of data in Ch. II with those of Braaksma *et al.* (5) shows that the decline in rate of e^--transport at redox potentials above -475 mV with flavodoxin is considerably more steep than is the case when using viologens or dithionite as e^--donors.

(In the latter case nitrogenase activity in redox-limited conditions follows a Nernst-type curve). Furthermore it appears that the redox potential above which the e^--donor becomes rate-limiting is considerably higher when using the artificial e^--donors; the rate becomes zero at approximately -370 mV, whereas with flavodoxin it becomes zero at approx. -460 mV. It is tempting, therefore, to assume that $FldH_2^{\cdot}$ has a multiple function to fulfill in switch-off. (a) $FldH_2^{\cdot}$ inhibits specifically the electron transport to nitrogenase above the critical $FldH_2^-/FldH_2^{\cdot}$ ratio, commensurate with a redox potential of -475 mV. That way, the e^--transport would be cut off more sharply and the function of the $FldH_2^-/FldH_2^{\cdot}$ ratio as a signal for intracellular O_2 would thus be strengthened. (b) However, if that signal is being given at a flavodoxin redox potential as low as -475 to -460 mV, and *if* that signal means that O_2 is actually present in the cell, and *if* flavodoxin under those conditions is still in redox equilibrium with nitrogenase, this would mean that nitrogenase would have to be protected against oxygen at a redox potential at which the separate nitrogenase components normally would be fully reduced. Results of Simpson and Burris (26) show that a mixture of Av_1, Av_2 and Fe/S II at that potential is hardly or not at all protected. The latter authors have, however, conducted their experiments in the absence of flavodoxin. So it is possible (though speculation) that $FldH_2^{\cdot}$ plays a second role in switch-off, namely, in the formation or stabilization of the oxygen-tolerant three-component complex even at potentials where the individual nitrogenase proteins and Fe/S II might still be reduced. Experimental data concerning this point are lacking; however it should be clear that only a *fast* formation of the complex would make physiological sense in conditions where Av_2 (and Av_1) need to be protected against O_2 breaking into the cell.

(2) It is an unanswered question whether Fe/S II should be oxidized simultaneously with Av_1 and Av_2 in order for complex to be formed; if so, how this would be accomplished. Perhaps significant to this point is the finding that nitrogenase might be located close to or associated with the cytoplasmic membrane (17): thus Fe/S II ($E_{m,7}$ = -225 mV, Scherings, unpubl. obs.) might easily be oxidized by e.g. cyt c ($E_{m,7}$ = +254 mV). Actually, Av_2 and Av_1 might prefer this pathway of oxidation (*via* Fe/S II to e.g. cyt c) to a presumably slower oxidation by H^+ or N_2. In any case, after oxidation by nitrogenase substrates they would have to be further oxidized since maximal protection is not reached until +15 mV (26). In Ch. III, data are presented showing that e^--transfer between Av_2 and Fe/S II is very efficient.

(3) In the scenario, a possible role of (Mg)ATP or (Mg)ADP has not been mentioned. Both are, however, important allosteric effectors of Av_2 and therefore certainly will influence formation of the oxygen-tolerant complex. Even more important perhaps is the role of free Mg^{2+}. If indeed, as Davis and Kotake (7) hypothesize, Mg^{2+} is released into the cytoplasm upon de-energization of the cytoplasmic membrane, this might enhance formation of the oxygen-tolerant complex (see further below). Therefore, it is quite to the point that, at the end of Ch. II, a scheme has been presented in which the roles of flavodoxin and Fe/S II have been incorporated into earlier proposals of Haaker (12) in which the nitrogenase activity was postulated to be dependent on the state of energization of the cytoplasmic membrane. Whatever molecular mechanism might actually be involved in the regulation of nitrogenase activity by the energizable membrane: proton gradient, membrane potential, regulation of the concentration of free Mg^{2+}, or ??, has been left open to the imagination of the reader.

Interlude

In the Introduction (I.2.3) the high respiration capacity of *A.vinelandii* has already been mentioned as a major reason why this organism with a true aerobic lifestyle is able at the same time to harbour the extremely O_2-sensitive nitrogenase system. However, on the other hand there are bacterial species with a respiration capacity that is as high or higher than that of *A.vinelandii* that do not fix nitrogen. It would be interesting to see whether the *nif*-genes plus the gene coding for Fe/S II could be transferred to such organisms (in a similar way as has been done in earlier transformations of *E.coli* (8) and *S.typhimurium* (24)) and still result in an active nitrogenase. Such experiments would seem a more logical preliminary to future attempts to genetically engineer nitrogen fixing cereals than premature attempts to transfer *nif*-genes into lower eukaryotes such as yeast (9)*.

Respiration in *A.vinelandii* has another important feature, namely, being highly adaptable to the concentration of O_2 in the environment. Oxygen uptake can

* In this context it is slightly surprising to note that in a talk on manipulations with *nif*-genes, given by Postgate and Cannon (23) at a plant breeding-oriented symposium, the word 'oxygen' was hardly mentioned at all! The authors suggested that for NIF-transformation of crop plants, the *chloroplast* genome (being akin to a prokaryotic-type genome) might be the site of choice. However, keeping in mind still the oxygen sensitivity of NIF, the *mitochondrial* genome (which possesses a prokaryotic-type of organization as well (11)) would appear at least as good a suggestion.

change several-fold without proportional change in ATP-production and without being inhibited by ATP. This 'oxygen-waste' (21) respiration is commonly viewed as a specific mechanism to protect nitrogenase from oxygen-inactivation without upsetting the cell's energy household too much. Thus it has been termed 'respiratory protection'. As mentioned in the Introduction (I.2.3 *c*) this adaptation mechanism is, however, unable to cope with sudden changes in external oxygen supply. A sudden change from low to high aeration (or removal of external C-sources at a constant oxygen tension) will result in oxygen entering the cell, and nitrogenase will consequently be 'switched-off'. The mechanism of 'switch-off' has been the implicit subject of chapters III and IV of this thesis.

The role of flavodoxin hydroquinone autoxidation in switching-off nitrogenase activity has already been indicated above. Without a continuous supply of reducing equivalents *via* the presumed physiological donor flavodoxin, nitrogenase will of course not function. (Whether *in vivo* nitrogenase is fully switched-off at -460 mV as is the case *in vitro* remains still to be seen!). Withdrawal of reducing equivalents cannot, however, explain the reversible character of the switch-off phenomenon, since oxidized nitrogenase proteins are just as oxygen-sensitive as the reduced ones (Scherings, unpubl. obs.).

A hypothesis has been offered in the above scenario. Below, evidence presented in Chapters III and IV will be shown to strengthen the concept of redox-dependent complex formation. Purified Av_1 and Av_2 will form a complex with Fe/S II which is as tolerant towards oxygen as a cell-free extract of *A.vinelandii*, -but only when they are oxidized and when a divalent cation (Mg^{2+}) is present.

Properties of the oxygen-tolerant nitrogenase complex

In Chapters III and IV, the formation *in vitro* of an oxygen-tolerant complex from the three purified components Avl_1, Av_2 and Fe/S II has been examined more closely. Findings may be summarized briefly as follows.

1. Both Av_1 and Av_2 separately are protected only slightly by Fe/S II against O_2; this in contrast to Av_1 and Av_2 together.
2. Mg^{2+} has a slight positive effect on the protection of Av_1 by Fe/S II and a negative effect on the protection of Av_2 by Fe/S II; protection of Av_1 + Av_2 however is strongly enhanced by Mg^{2+}.
3. Formation of a three-component complex is redox-dependent and promoted by Mg^{2+}.

4. By mixing the three oxidized components, in the presence of 5 mM $MgCl_2$, a complex is obtained which has no narrowly fixed stoichiometry. It is possible to isolate high-molecular mass fractions from such a mixture; these do not behave homogeneously in the ultracentrifuge but have their S-values centered around 34 S. A tentative estimation of the most probably stoichiometries indicates that the molecular composition of a complex might be within these limits: 4 molecules of Av_1 versus 8-12 molecules of Av_2 and 4-6 molecules of Fe/S II. Another stoichiometry or molecular mass however may be just as tolerant to oxygen: e.g. in the presence of MgADP a complex of lower molecular mass is obtained (approx. 0.4 MDa) without this having an effect on oxygen tolerance.

Naturally, no definite proof can be given here that the high-molecular mass three-component complex(es) described in Ch. IV really represent(s) the physiological switched-off nitrogenase. Apart from possible effects of MgADP or MgATP, also the initial concentrations of the separate components obviously would affect the formation of a stable complex. Whatever the physiological relevance, the complexes as isolated appear interesting enough to warrant a study in their own right. Two questions obviously demand an answer. (1) What is the *mechanism* of redox- and Mg^{2+}-dependent assembly/dissembly of the complex, and is it quick enough to prevent damage *in vivo*? In Ch. III some preliminary results have been mentioned concerning possible pathways of electron transport within the three-component complex. (2) What is the *mechanism* of protection against oxygen inactivation. Studies on the structure of the complex, e.g. by cross-linking or crystallization followed by X-ray diffraction studies, might provide some insight. Such studies have not been initiated as yet.

Concluding remarks

The latter question of course is directly linked to the big, as yet unanswered question: what is the mechanism of oxygen toxicity in nitrogenase anyway? It is noteworthy that hardly any work has been devoted towards a study of the O_2-sensitivity of these proteins. The feeling about the property referred to here is usually merely one of annoyance. However, in other fields of biology, awareness has been growing for some time that O_2 and O_2-derivatives are potentially harmful agents in all biological systems. Also, the property of O_2-sensitivity might turn out to be a useful probe of other properties of the

Fe-protein of nitrogenase.

Often, when dealing with oxygen toxicity in biological systems, peroxide or superoxide radicals are invoked (10): also with oxygen toxicity in nitrogenase of *C.pasteurianum* this has been the case (25). With Av_2, however, no effect of either SOD, catalase, or radical scavengers has been observed on the rate of inactivation by oxygen (Scherings, unpubl. obs.). The explanation for such lack of exact data is probably quite simply this: the technical difficulties involved. Firstly, reactions with gases in solution are of course difficult to control; and secondly how and what to measure. Measuring 'remaining specific activity' at set intervals during the reaction is -as anyone who has ever made the attempt will know- excruciatingly tedious and time-consuming; secondary parameters like absorbance changes or oxygen uptake which are much more easy to follow during the reaction appear not to have any obvious kinetic relationship with remaining specific activity (Scherings, unpubl. obs.). If loss of specific activity is exclusively due to damage to the Fe-S clusters, physical studies (E.P.R., Mössbauer spectroscopy) obviously would be the method of choice.

Technical problems should not deter anyone from tackling the problem anyway. Oxygen protection of nitrogenase IS, after all, an issue of overriding importance if nitrogen fixation research is to have any future impact on agriculture.

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SAMENVATTING

Alle in dit proefschrift beschreven experimenten hebben betrekking op de C-heterotrofe, N-autotrofe, obligaat aerobe, vrij in de bodem levende bakterie *Azotobacter vinelandii*.
Aan de basis van dit proefschrift, zoals aan die van de eerder verschenen proefschriften van Haaker (3) en Laane (4) die eveneens op het Laboratorium voor Biochemie te Wageningen zijn bewerkt, liggen een viertal eenvoudig verifieerbare fenomenen zoals die bij *A.vinelandii* zijn waar te nemen.

1. Stikstofbinding is afhankelijk van extern aangeboden O_2; een kurve die de snelheid van stikstofbinding weergeeft in afhankelijkheid van de externe concentratie O_2 (ceteris paribus) heeft de typische klokvorm ('bell-shape'), en vertoont dus een optimum.
2. De ligging van bovengenoemd optimum is afhankelijk van het zuurstofaanbod tijdens groei; de opnamesnelheid van bepaalde ademhalingssubstraten zomede de samenstelling van de ademhalingsketen blijkt genetisch reguleerbaar onder invloed van O_2.
3. Op snelle (te snel voor eiwitsynthese) verhoging van het zuurstofaanbod reageert het organisme met 'switch-off', d.i. een momentane, reversibele inaktivatie van het stikstoffixerend systeem.
4. De twee geïsoleerde eiwitkomponenten van het stikstoffixerend enzym (Av_1 en Av_2) worden snel en irreversibel geïnaktiveerd door O_2.

Het is duidelijk dat de relatie O_2-N_2 centraal staat bij de stikstofbinding door *A.vinelandii*. In de wetenschap dat de nitrogenase eiwitten *per se* tot 10% van het totaal aan oplosbare eiwitten kunnen uitmaken in een stikstofbindend organisme, kunnen we wellicht zelfs stellen dat die relatie centraal staat in het metabolisme als geheel.
Haaker en Laane, in hun proefschriften, zijn uitgebreid ingegaan op met name de punten 1 en 2: de rol die het cytoplasma-membraan, en speciaal de ademhalingsketen, zou spelen in het proces van stikstoffixatie. Dit proefschrift beschrijft experimenten die relevant zijn voor de punten 3 en 4. In tegenstelling tot de proefschriften van beide eerder genoemde auteurs worden hier experimenten beschreven met *geïsoleerde* komponenten van het stikstofbindingssysteem. Wel is de fysiologische relevantie zoveel mogelijk in het oog gehouden.

Hoofdstuk II beschrijft een aantal aspekten van de nitrogenase aktiviteit van een eiwit-kompleks dat *als zodanig* uit een celvrij extrakt van *A.vinelandii*

kan worden geïsoleerd. Dit kompleks bevat naast Av_1 en Av_2 een aantal 'kontaminerende' eiwitten (zie onder). In plaats van dithioniet is flavodoxine gebruikt als (waarschijnlijk fysiologische) elektronendonor. Er blijken dan een aantal opvallende verschillen te zijn met de standaard-nitrogenase reaktie zoals die wordt gemeten aan gezuiverde nitrogenase komponenten (Av_1 + Av_2) met dithioniet als e^--donor. Ze kan als volgt gekarakteriseerd worden.

(a) *A.vinelandii* flavodoxine wisselt tijdens steady-state electronentransport naar nitrogenase de hydrochinon vorm ($FldH_2^-$) af met de semichinon vorm ($FldH_2^{\cdot}$); de chinon vorm speelt geen rol.

(b) De maximumsnelheid van electronentransport ligt hoger dan bij gebruik van dithioniet als e^--donor, en is afhankelijk van de initiële verhouding $FldH_2^-/FldH_2^{\cdot}$ ('startpotentiaal').

(c) De oxidatie van substraathoeveelheden $FldH_2^-$ door H^+, via het ruwe *A.vinelandii* nitrogenase kompleks, vertoont in de tijd drie karakteristieke fasen: (1) een initiële lagfase, (2) een konstante snelheid bij afnemende $FldH_2^-/FldH_2^{\cdot}$ verhouding totdat die verhouding gedaald is tot een waarde overeenkomend met een berekende redoxpotentiaal van ca. -475 mV, en (3) boven -475 mV een zeer steile afname van de oxidatiesnelheid tot 0 bij ca. -460 mV.

Het bleek mogelijk de lagfase teniet te doen door één van de 'kontaminerende' eiwitten, het zgn. Fe/S eiwit II, te verwijderen uit het ruwe kompleks. Fe/S II is een [2Fe-2S]-type ferredoxine. Echter, de lagfase bleek óók verdwenen te zijn als het oorspronkelijke kompleks fotochemisch gereduceerd werd alvorens de reaktie te starten! Deze waarneming gaf aanleiding te postuleren dat het fenomeen 'switch-off' zoals men dat bij hele cellen kan waarnemen, op molekulair nivo verklaard zou kunnen worden uit een *redox-afhankelijke kompleksvorming* van de drie belangrijkste eiwitkomponenten uit het ruwe kompleks: Av_1, Av_2 en Fe/S II. De redoxtoestand van het koppel $FldH_2^-/FldH_2^{\cdot}$ zou daarbij als 'trigger' dienen voor kompleksassociatie/dissociatie.

Scenario

Niet vergeten mag worden dat het hier slechts een werkhypothese betreft, -al was het alleen maar vanwege het feit dat niet met 100% zekerheid vaststaat dat flavodoxine, en flavodoxine alleen, de fysiologische e^--donor voor nitrogenase in *A.vinelandii* is. Toch is ze door haar eenvoud bevredigend en fysiologisch relevant. Men kan zich deze opeenvolgende gebeurtenissen voorstellen:

(a) Het zuurstofaanbod nadert de grens van de gegeven ademhalingskapaciteit in een *A.vinelandii* cel en stijgt. (b) Beschikbare reduktie-equivalenten worden in het kader van de ademhalingsbescherming van nitrogenase zoveel mogelijk afgeleid naar O_2; de ademhalingsketen raakt geoxideerd; kortom de beschikbare 'reducing power' daalt beneden het nivo dat nodig is om flavodoxine semichinon nog te kunnen reduceren. (c) De redoxpotentiaal van het koppel $FldH_2^-/FldH_2^\cdot$ nadert -475 mV en stijgt. (d) O_2 diffundeert de cel binnen. (e) $FldH_2^-$ wordt nu zeer snel door O_2 geoxideerd. De elektronenstroom naar nitrogenase is daarmee afgesneden. (f) Av_1 en vervolgens Av_2 worden geoxideerd door N_2 en H^+ en vormen een inaktief, zuurstof-tolerant kompleks met Fe/S II.

Bij het laatste onderdeel van bovenstaand scenario passen een aantal kanttekeningen.

(1) Het is een onbeantwoorde vraag of Fe/S II tegelijkertijd met Av_1 en Av_2 geoxideerd moet raken om een zuurstoftolerant kompleks te kunnen vormen; en zo ja, hoe dat in z'n werk gaat.

(2) Flavodoxine semichinon zou een specifieke rol kunnen spelen bij de vorming van het zuurstof-tolerante kompleks, en wel om de volgende redenen. Vergelijking van in hoofdstuk II vermelde resultaten met resultaten van Braaksma *et al.* (1) geeft aan dat de daling in snelheid van e^--transport boven een redoxpotentiaal van -475 mV aanzienlijk steiler is dan het geval is bij gebruik van viologenen of dithioniet als e^--donor. (In het laatste geval, dus met gereduceerd viologeen of dithioniet als snelheidslimiterende e^--donor, blijkt de nitrogenase aktiviteit vrij netjes beschreven te kunnen worden door middel van de Nernst-vergelijking). Bovendien blijkt bij gebruik van deze artificiële e^--donoren de redoxpotentiaal waarboven de e^--donor snelheidbeperkend wordt, aanzienlijk hoger te liggen; de snelheid wordt pas 0 bij ca. -370 mV in tegenstelling tot het geval bij flavodoxine als e^--donor, nl. ca. -460 mV. Het is daarom verleidelijk om aan te nemen dat $FldH_2^\cdot$ meerdere funkties vervult bij switch-off. (a) $FldH_2^\cdot$ remt specifiek het elektronentransport naar nitrogenase boven de kritische $FldH_2^-/FldH_2^\cdot$ verhouding die overeenkomt met een redoxpotentiaal van -475 mV. Het e^--transport zou hierdoor verscherpt worden 'afgekapt' en de funktie van de $FldH_2^-/FldH_2^\cdot$ verhouding als een signaal voor intracellulair O_2 worden versterkt. (b) Als dat signaal echter reeds wordt gegeven bij een flavodoxine redoxpotentiaal van ergens tussen -475 en -460 mV, en *als* dat signaal inderdaad betekent dat O_2 in de cel aanwezig is, en *als* flavodoxine dan nog steeds in redox-evenwicht is met nitrogenase, moet nitrogenase dus

reeds tegen O_2 worden beschermd bij een redox-potentiaal waarbij de afzonderlijke komponenten normaliter vrijwel volledig gereduceerd zouden zijn. Resultaten van Simpson en Burris (5) tonen aan dat een mengsel van Av_1, Av_2 en Fe/S II bij die potentiaal niet of nauwelijks beschermd is. Laatstgenoemde auteurs hebben hun experimenten echter uitgevoerd in afwezigheid van flavodoxine. Het is dus denkbaar (hoewel speculatie) dat $FldH_2^{\cdot}$ een tweede rol vervult bij switch-off, en wel bij de vorming of stabilisatie van het zuurstof-tolerante drie-komponenten kompleks. Experimentele gegevens hierover ontbreken; het is echter duidelijk dat alleen een *snelle* vorming van het kompleks fysiologisch zinnig zou zijn als het erom gaat Av_2 (en Av_1) te beschermen tegen de cel binnendringend O_2.

(3) In het scenario is een eventuele rol van (Mg) ATP of (Mg)ADP onbesproken gebleven. Beide zijn echter belangrijke allostere effektoren van Av_2 en zullen dus zeker de vorming van het zuurstof-tolerante kompleks beïnvloeden. Belangrijker nog is wellicht de rol van vrij Mg^{2+}. Als deënergisatie van het cytoplasma membraan inderdaad leidt tot afstoot van cationen naar het cytoplasma, zoals Davis en Kotake (2) veronderstellen, zou dit de vorming van het zuurstof-tolerante kompleks kunnen versnellen (zie onder). Om deze reden is dan ook aan het slot van hoofdstuk II een schema gepresenteerd waarin de rol van flavodoxine en die van Fe/S II bij switch-off en switch-on zijn ingebed in eerdere voorstellen van Haaker (3), waarbij de nitrogenase aktiviteit in *A.vinelandii* afhankelijk is gesteld van de energisatie-toestand van het cytoplasma membraan. In het midden gelaten is, via welke molekulaire mechanismen de energisatie-toestand van belang zou kunnen zijn bij aktiviteit en regulatie van de nitrogenase aktiviteit: protonengradiënt, regulatie van de concentratie vrij Mg^{2+}, of ??.

Eigenschappen van het zuurstof-tolerante nitrogenase kompleks

In de hoofdstukken III en IV is de vorming *in vitro* van een zuurstof-tolerant kompleks uit de drie gezuiverde komponenten Av_1, Av_2 en Fe/S II nader onderzocht. De bevindingen kunnen als volgt beknopt worden samengevat.

1. Zowel Av_1 als Av_2 worden ieder apart in slechts geringe mate door Fe/S II beschermd tegen O_2; dit in tegenstelling tot Av_1 en Av_2 tezamen.
2. Mg^{2+} heeft slechts een gering positief effekt op de bescherming van Av_1 door Fe/S II en zelfs een negatief effekt op de bescherming van Av_2; bescherming van Av_1 + Av_2 door Fe/S II wordt evenwel sterk bevorderd door Mg^{2+}.

3. De vorming van een drie-komponenten kompleks is redox-afhankelijk en wordt door Mg^{2+} bevorderd.
4. Menging van Av_1, Av_2 en Fe/S II in geoxideerde toestand, in aanwezigheid van 5 mM $MgCl_2$, levert kompleks op van een niet nauwkeurig gedefinieerde stoichiometrie. De uit zo'n mengsel geïsoleerde frakties van hoog-molekulaire massa gedragen zich in de ultracentrifuge niet homogeen, maar met S-waarden gecentreerd rond 34 S. Een voorlopige schatting van meest waarschijnlijke stoichiometrieën geeft aan dat de molekulaire samenstelling van een kompleks zich binnen deze grenzen zou kunnen bevinden: 4 molekulen Av_1, 8-12 molekulen Av_2, en 4-6 molekulen Fe/S II. Een andere samenstelling qua stoichiometrie of molekulaire massa kan echter even zuurstof-tolerant zijn: b.v. in de aanwezigheid van MgADP wordt een kompleks van lagere molekulaire massa gevormd (ca. 0.4 MDa) zonder dat dit een effekt heeft op de zuurstof-tolerantie.

Zoals uit het laatstgenoemde blijkt, is nog onduidelijk of een 34 S kompleks ook *in vivo* wordt gevormd. Naast effekten van MgADP of MgATP blijken ook de uitgangsconcentraties van de afzonderlijke komponenten van belang te zijn bij de vorming van een stabiel kompleks. Technische moeilijkheden, vooral bij de isolatie van zuiver kompleks, vrij van de afzonderlijke komponenten (vnl. gepolymeriseerd Av_1), bemoeilijken het onderzoek. Kristallisatie zou een oplossing kunnen zijn en bovendien struktuuronderzoek mogelijk maken.

Literatuur

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CURRICULUM VITAE

Schrijver dezes aanschouwde het levenslicht op 11 juni 1949 te Groningen. In 1968 behaalde hij het gymnasium B diploma aan het Heymans Lyceum te Groningen. In datzelfde jaar begon hij zijn studie aan de Landbouwhogeschool te Wageningen. In 1973 werd het kandidaatsexamen Akker- en Weidebouw behaald en in 1975 het doctoraalexamen (hoofdvakken biochemie en plantenfysiologie, bijvak landbouwplantenteelt).

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