ELECTRON TRANSPORT TO NITROGENASE IN *AZOTOBACTER VINELANDII*

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Proefschrift

ter verkrijging van de graad van
doctor in de landbouwwetenschappen,
op gezag van de rector magnificus,
dr. C.C. Oosterlee,
in het openbaar te verdedigen
op woensdag 4 december 1985
des namiddags te vier uur in de aula
van de Landbouwhogeschool te Wageningen

Voor Hilde

STELLINGEN

1. De conclusie van Estell en medewerkers, dat subtilisine, door middel van plaatsgerichte mutagenese stabieler gemaakt tegen oxidatie-middelen, bruikbaar zou zijn voor industriële toepassingen, is voorbarig.
   - Estell, D.A., T.P. Graycar en J.A. Wells (1985) Journal of Biological Chemistry 260, 6518-6521.
   - Bott, R.R., E. Ferrari, J.A. Wells, D.A. Estell, D.J. Henner (1985) European patent application 0130756 (79 pp.).

2. De resultaten verkregen in 26 verschillende laboratoria van een kwantitatieve analyse van soja-eiwit in verschillende preparaten met SDS-PAGE en met ELISA doen vermoeden, dat Western blotting gevolgd door immunologische detectie een nauwkeuriger methode is voor de bepaling van soja-eiwit in vleesprodukten.
   - Olsman, W.J., S. Dobbelaere en C.H.S. Hitchcock (1985) Journal of the Science of Food and Agriculture 36, 499-507.

3. De methode gebruikt door Jouanneau en medewerkers is ongeschikt voor het bepalen van de concentratie van nitrogenase in hele cellen.
   - Jouanneau, Y., B. Wong en P.M. Vignais (1985) Biochimica et Biophysica Acta 808, 149-155.
   - Dit proefschrift, hoofdstuk 3.

4. Gezien de nauwkeurigheid van de resultaten kan in veel biochemische publikaties een grafiek vaak beter met een viltstift dan met een pen getekend worden.

5. De remming van de stikstofbinding, die wordt waargenomen in Anabaena na het toevoegen van NaCl kan beter verklaard worden in termen van een effect van NaCl op de proton motive force, dan door remming van de nitrogenase activiteit via een verhoogde interne Na^+ concentratie.
   - Moore, D.J., R.H. Reed en W.D.P. Stewart (1985) Journal of General Microbiology 131, 1267-1272.

6. Wanneer een Coomassie Brilliant Bue eiwitbepaling wordt vergeleken met de biureet of de Lowry methode, kan een uitspraak gedaan worden over het gehalte aan basische aminozuren.
   - Tal, M., A. Silberstein en E. Nusser (1985) Journal of Biological Chemistry 260, 9976-9980.

7. De referees van het betreffende artikel geven blijk van een gebrekkige kennis van de literatuur, wanneer zij accepteren dat Schägger en medewerkers een "nieuwe" SDS-PAGE methode ontwikkeld hebben om 11 eiwitten met een molecuulmassa van 6 tot 50 kDa te scheiden.
   - Schägger, H., U. Borchart, H. Aquila, T.A. Link en G. von Jagow (1985) FEBS Letters 190, 89-94.
   - Burr, F.A. en B. Burr (1983) Methods in Enzymology 96, 239-244.

8. Kennis is macht. De onmacht van gehandicapten maakt het daarom gemakkelijk om bezuinigingen op hen af te wentelen.
   - Eigen bijdrage regeling bijzondere ziektekosten 1983, Nederlandse Staatscourant, ma. 8 november 1982, nr. 215.

9. Bij het vrijmaken van ijzer uit ferritine is de pH belangrijker dan de redoxpotentiaal.
   - Funk, F., J.-P. Lenders, R.R. Crichton en W. Schneider (1985) European Journal of Biochemistry 152, 167-172.

10. Uit de gegevens die Ramos & Robson publiceren over de ademhaling en de stikstofbinding van Azotobacter chroococcum kan afgeleid worden, dat het electronentransport naar nitrogenase in deze bacterie vijf maal effectiever is dan in Azotobacter vinelandii.
   - Ramos, J.L. & R.L. Robson (1985) Journal of Bacteriology 162, 746-751.
   - Dit proefschrift, hoofdstuk 5.

11. De term neuron specifiek voor de iso-enzymen van enolase, die de γ-subeenheid bevatten, is onjuist.
   - Oskam, R., Rijksen, G., Lips, C.J.M. en Staal, G.E.J. (1985) Cancer 55, 394-399.

12. Kleuters zijn uitermate geschikt als adviseurs voor reclamebureaus, aangezien uit hun reactie gemakkelijk valt af te leiden welke STER reclames pakkend zijn.

13. De meeste stellingen worden in de bouw gezet.

Jan Klugkist
Electron transport to nitrogenase in Azotobacter vinelandii
Wageningen, 4 december 1985

VOORWOORD

Bij dezen wil ik iedereen bedanken, die heeft bijgedragen aan het tot stand komen van dit proefschrift.

In de eerste plaats Dr. Huub Haaker, die het onderzoek op zeer bekwame wijze begeleid heeft. Zijn manier van werken heb ik steeds als bijzonder plezierig en inspirerend ervaren.

Mijn promotor, Prof.dr. Cees Veeger ben ik dankbaar voor het feit, dat hij in een tijd van bezuinigingen steeds weer de financiële middelen voor het onderzoek wist te verkrijgen.

Doordat er met hen altijd wel wat te beleven viel hebben Michiel Appels, Arnold Braaksma, Jan Cordewener en Hans Wassink gezorgd voor een uitermate prettige werksfeer op lab. III.

Ton van Berkel en Jan Voorberg wil ik bedanken voor het aandeel wat zij als doctoraalstudenten in het onderzoek hebben gehad.

Margreet Krüse-Wolters wil ik bedanken voor haar hulp bij de allerlaatste experimenten.

De heer J. Haas en medewerkers van het centrum kleine proefdieren hebben op uitstekende wijze de konijnen verzorgd, die gebruikt zijn voor de antilichaamproductie.

Ton Bisseling, Rommert van den Bos en Jan Hontelez hebben steeds als vraagbak willen fungeren voor de moleculair biologische kant van het onderzoek. Jenny Toppenberg-Fang heeft het manuscript uitgetypt en Martin Bouwmans heeft het tekenwerk verzorgd. Het fotografische werk is gedaan door Piet Madern. Ook hen wil ik bedanken.

Tot slot wil ik een aantal personen noemen, die niet direct met dit proefschrift te maken hebben, maar die zo onlosmakelijk met mijn leven verbonden zijn, dat ik ook hen in mijn dankwoord wil betrekken. De God, die mijn Schepper is, mijn Redder en mijn Heer. Mijn ouders, voor hun opvoeding en hun levendige belangstelling voor mijn studie. Mijn vrouw Klasien, voor de inzet waarmee ze ons huwelijk steeds weer fris weet te houden.

CONTENTS

LIST OF ABBREVIATIONS

A_{272}	absorbance at 272 nm
Ac	acetate
ATCC	American type culture collection
Av_1	MoFe-protein of nitrogenase of Azotobacter vinelandii
Av_2	Fe-protein of nitrogenase of Azotobacter vinelandii
Da	dalton = mass of one hydrogen atom
E_m	midpoint redox potential at pH 7.0 and 25°C
E_h	observed redox potential of a system
E1	midpoint potential for the redox couple flavodoxin semiquinone/flavodoxin hydroquinone
E2	midpoint potential for the redox couple flavodoxin quinone/flavodoxin semiquione
EDTA	ethylenediaminetetraacetate
EPR	electron paramagnetic resonance
Fd	ferredoxin
Fld	flavodoxin of Azotobacter vinelandii
FMN	riboflavin 5'-phosphate
FPLC	fast protein liquid chromatography
Fe/S II	iron-sulfur protein II of Azotobacter vinelandii
M_r	relative molecules mass
nif	nitrogen fixation
NMR	nuclear magnetic resonance
nod	nodulation
pI	iso-electric point
PMSF	phenylmethylsulfonyl fluoride
SDS	sodium dodecylsulfate
Tes	N-tris(hydroxymethyl)methyl-2-amino-ethanesulfonic acid
Tris	tris(hydroxymethyl)aminomethane
$\Delta\tilde{\mu}_{H^+}$	proton motive force
$\Delta\Psi$	transmembrane electrical potential
ΔpH	transmembrane pH gradient.

1. GENERAL INTRODUCTION

1.1 The nitrogen cycle

Nitrogen is a component element of proteins, nucleic acids and other biomolecules. Biological structures in plant and animal kingdoms contain only 1/400000 of the nitrogen present in the atmosphere [18,24], but cannot use the atmospheric nitrogen (N_2) for their growth. The conversion of dinitrogen to ammonia is carried out by some bacteria and blue-green algae, a process called nitrogen fixation. The ammonia can be incorporated into plant material. Plants are consumed by animals. As a result of decay and putrefication of plant and animal material, ammonia returns to the soil, where bacteria rapidly convert it into nitrate (nitrification). Nitrate can either be incorporated into living material e.g. used by plants or return to the atmosphere after denitrification by bacteria. The last step closes the nitrogen cycle as shown in Fig. 1.

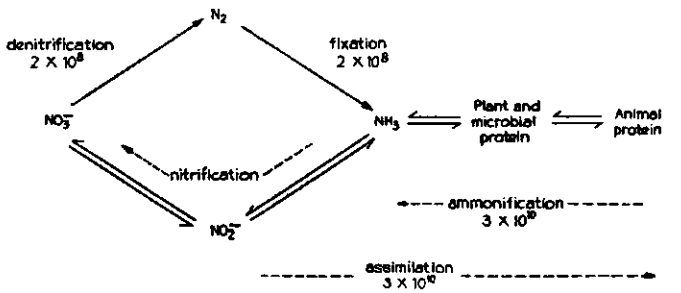

Fig. 1. The nitrogen cycle. The simplest form of the nitrogen cycle is illustrated. The numbers beneath the various steps are orders of magnitude of turnover in tonnes/year. From ref. 64.

1.2 Biological nitrogen fixation

Much of the N-cycling occurs at the lower part of the cycle, assimilation and ammonification (Fig. 1). Input of inorganic-N by nitrogen fixation is about 1% of the assimilation rate. In nature N_2-fixation controls the flux of N through the N-cycle [40]. At low levels of fixed-N nitrogen fixation is induced. When fixed-N is relatively abundant, nitrogen fixation stops to prevent that biomass will increase indefinitely.

In practice productivity of agriculture is often limited by the availability of fixed-N. Therefore intensive agriculture requires the input of inorganic nitrogen fertilizers. Ammonia necessary for these fertilizers is made industrially from hydrogen gas and atmospheric nitrogen in the Haber-Bosch process. Industrial fixation amounts to approximately one fifth of the total nitrogen fixation in the world [14]. Up till now industrial fixation is relatively cheap, but the fossil fuels needed for the process are exhaustible. Furthermore, even though nitrogenous fertilizers are cheap, the costs of transport makes the price of fertilizer a great obstacle to increase food production in many less developed countries. So it is not amazing that since long there is a great interest in biological nitrogen fixation. During the last decade scientific interest has been expanding. The first international symposium on nitrogen fixation in 1974 was attended by 200 scientists. The fifth sysmposium in 1983 by 550 scientists from 60 different countries. Strange enough increase in food production is mainly expected from increase in input of (biologically) fixed-N. Decreasing loss of fixed nitrogen by denitrification (see Fig. 1) could also be beneficial for agriculture. But relatively little attention is given to this point [63].

How biological nitrogen fixation might attribute to the worlds food problem is reflected by the research presented at conferences on N_2-fixation [88]. Firstly, attention is given to the application of biological nitrogen fixation. Often environmental stresses prevent the presently available, active nitrogen-fixing microorganisms from doing what laboratory and greenhouse studies suggest they ought to do. Identifying these stresses

should facilitate the development of nitrogen-fixers that can survive, grow, and be beneficial in agricultural environments [1].

Secondly, an understanding of the chemistry of dinitrogen fixation might lead to the development of more effective catalysts than are used at present in the Haber-Bosch process, needing a N_2-pressure of 300 atmospheres and a temperature of 500°C.

Thirdly, the enzyme that actually 'fixes' N_2 i.e. nitrogenase is studied to figure out the catalytic mechanism of biological nitrogen fixation.

Fourthly, physiology is an important, but a broad research area. It deals with questions as: how is the process of nitrogen fixation incorporated into the metabolism of bacteria; how is the process regulated; what is necessary for the development of an effective symbiosis between nitrogen-fixing organisms and plants.

Fifthly, genetists are isolating and manipulating the (nif) genes, involved in nitrogen-fixation, from bacteria. Although it is clear that 'nitrogen-fixing' cereals are not simply made by transferring the nif-genes to the plant of choice, the nif-genes might be used in transforming non-nitrogen-fixing bacteria living in associations with crops into diazotrophs to the benefit of the plants. Another line of research deals with the genetics of symbiotic nitrogen fixation. This includes identification of bacterial genes necessary for nodulation (nod-genes) as well as the many plant genes involved in nodulation and symbiosis. The apparent aim of this kind of research is to obtain the basic knowledge in order to construct new kinds of symbiosis between nitrogen-fixing bacteria and commercially important crops. Developing, for example, corn plants having symbiotic nitrogen fixation as pea plants do. Maybe on the long term, important agricultural crops can be engineered to fix their own nitrogen without the involvement of bacteria [57], but at this moment many genetical, biochemical, physiological problems are unsolved.

1.3 Enzymology

For all organisms studied thus far the properties of the enzyme system nitrogenase, which catalyzes the reduction of N_2 to ammonia are remarkably similar [13,32]. In nearly all diazotrophs biosynthesis of nitrogenase is repressed, when cells are provided with fixed nitrogen. Thus nitrogen fixation occurs only when its product (NH_3) is needed. Nitrogenase is a complex of two proteins, neither of which has any activity by itself. The two proteins can be purified separately and are called molybdenum-iron protein (MoFe protein or component I) and iron protein (Fe protein or component II). The MoFe protein is an $\alpha_2\beta_2$ tetramer of relative molecular mass (Mr) 220000 [50]. The α subunit (Mr 50000) is coded by the so-called *nif* D gene. The β subunit (Mr 60000) is coded by the *nif* K gene. The MoFe protein is thought to have 2 iron-molybdenum cofactors (FeMoCo) as the substrate binding sites [78]. One FeMoCo consists of 1 atom of Mo, 8 Fe atoms and 6 S^{2-} atoms [20,75]. In addition the MoFe protein contains about 4 [4Fe-4S] clusters.

The Fe protein is a dimer composed of two identical subunits, encoded by the so-called *nif* H gene. The native Fe protein, of Mr 65000, is generally thought to contain one [4Fe-4S] cluster and two adenine nucleotide binding sites [13]. Binding of MgADP and MgATP to the Fe protein is thought to be competative [15]. As discussed by Haaker and Veeger [32], EPR and Mössbauer studies indicate that only a minor part of the Fe atoms in the Fe protein of *Azotobacter vinelandii* (Av2) are present in a ferredoxin type of [4Fe-4S] cluster. Most of the iron atoms are part of another cluster which has not been detected by EPR spectroscopy. The exact number of Fe and S^{2-} atoms per molecule Fe protein is still unknown. Although most authors report a value of 4 [13], Braaksma *et al.* [12] showed that the number was variable between 3 and 8 dependable upon the growth conditions of the bacteria and upon the isolation procedure. Up to now no unambiguous biochemical data about the cluster content of the MoFe and the Fe protein can be given. This is caused by the fact that it is very difficult to purify these proteins without loss of activity or loss of metal clusters.

So one is never sure, that experiments are done with preparations similar to the proteins as they occur *in vivo*.

The stoichiometry of the reaction catalyzed by nitrogenase under optimum conditions is [79]:

$$N_2 + 10H^+ + 8 \text{ electron donor (red)} + 16 \text{ MgATP} \rightarrow$$

$$2 NH_4^+ + H_2 + 8 \text{ electron donor (ox)} + 16 \text{ MgADP} + 16 P_i \quad \text{(eq.1)}$$

Under less favourable conditions the amount of ATP hydrolyzed and the amount of H_2 produced per molecule of NH_4^+ formed is higher. The enzyme also reduces acetylene to ethylene, a compound which can rapidly and sensitively determined by gas chromatography and is therefore used in many biochemical studies. Because nitrogenase is rapidly inactivated by oxygen, all nitrogen-fixers have provisions to keep the environment of the enzyme anaerobic [6]. Anaerobic, facultative anaerobic and phototrophic bacteria only fix nitrogen under anaerobic conditions. Aerobic bacteria protect nitogenase against oxygen by physical barriers, a high respiration rate and in the case of *Azotobacter* species an FeS protein which binds to nitrogenase and protects it against oxygen damage after a sudden increase in aeration. A detailed review about the maintenance of anaerobiosis inside aerobic nitrogen-fixing bacteria is given by Scherings [70].

1.4 *Electron transport to nitrogenase*

For nitrogenase activity the electron donor shown in equation 1 must have a redox potential of -400 mV or lower [11,21,49,71]. *In vitro* sodium dithionite is almost exclusively used as reducing agent. *In vivo* only two classes of electron carriers with sufficiently low redox potential are known, namely ferredoxins and flavodoxins.

Ferredoxins are proteins with an FeS cluster as prosthetic group and M_r ranging from 5600 to 24000 [27,92,93,95]. For cyanobacteria the prosthetic group is a [2Fe-2S] cluster. All other low potential ferredoxins from

nitrogen-fixing organisms have a [4Fe-4S] cluster (Fig. 2). From many bacteria two or more ferredoxins can be isolated. Another feature, thus far only observed in nitrogen-fixing bacteria, is that some ferredoxins have two [4Fe-4S] clusters operating at two different redox potentials. One cluster having an E_m of ca. -400 mV, the other being +300 mV. Recently it has been found, that one of the clusters is easily converted into a [3Fe-3S] cluster and vice versa [58,59]. Ferredoxin is considered to be the immediate electron carrier to nitrogenase in all nitrogen-fixing organisms, with the exception of Klebsiella pneumoniae and possibly Azotobacter species. Under Fe-deficiency several microorganisms form flavodoxin instead of ferredoxin [8,16,19,44]. Only for Klebsiella pneumoniae [94] and Azotobacter vinelandii [86] synthesis of flavodoxin was shown not to be supressed by high concentrations of iron in the medium.

Flavodoxins are soluble proteins having FMN as prosthetic group (Fig. 2) and M_r ranging from 14000 to 23000 [27,92,93,95]. Flavodoxins have three oxidation levels: quinone (oxidized form), semiquinone ($1e^-$-reduced) and hydroquinone ($2e^-$-reduced). The redox potential of the hydroquinone/semiquinone couple is sufficiently low to reduce nitrogenase [27,92,93,95].

The physiological flow of electrons is thought from either ferredoxin or flavodoxin to the Fe protein of nitrogenase. The Fe protein in turn reduces the MoFe protein coupled to MgATP hydrolysis. When 8 electrons are transferred to the MoFe protein, one molecule of N_2 can be reduced completely, a process requiring both the MoFe protein and the Fe protein. As will be discussed in the next sections, little is known about the way the electron carriers ferredoxin and flavodoxin are reduced in vivo.

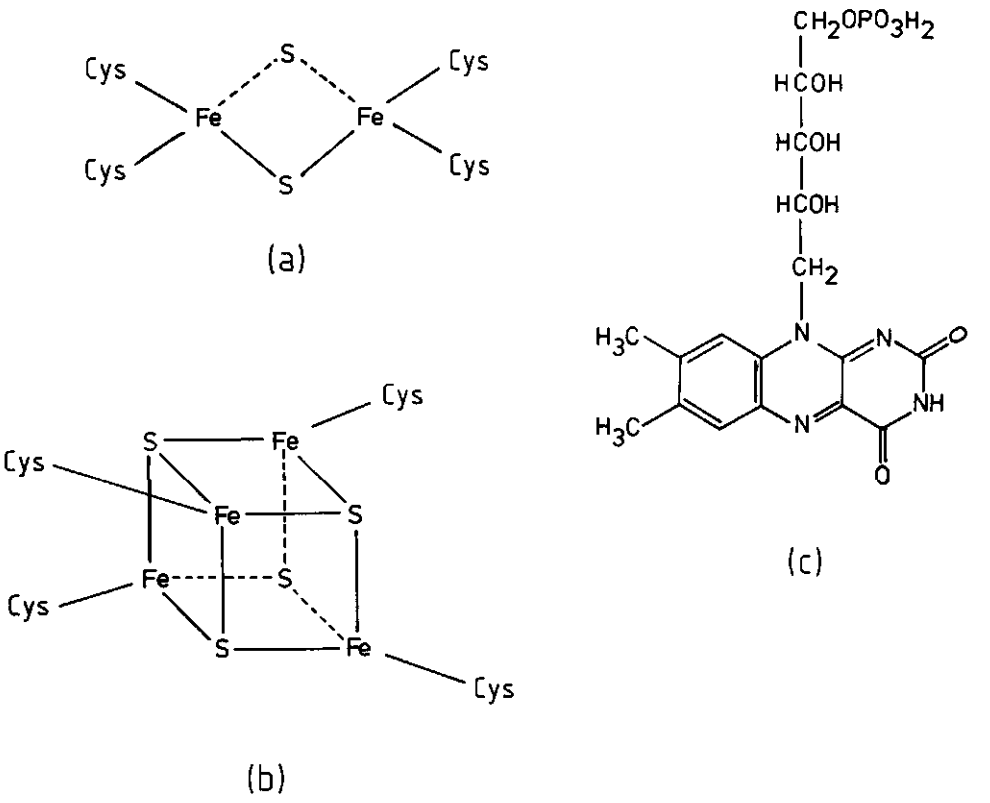

Fig. 2. Arrangement of iron and sulphur atoms in (a) [2Fe-2S] and (b) [4Fe-4S] clusters. The cysteine residues are part of the polypeptide chain of the ferredoxin and are bound to the clusters via their S atoms. (c) Structure of oxidized riboflavin 5'-phosphate (FMN).

1.4.1 Obligate anaerobic nitrogen-fixing bacteria

This group of bacteria includes the obligate anaerobes Clostridium pasteurianum and Desulfovibrio spp. but also the facultative anaerobes like Klebsiella pneumoniae and Bacillus polymyxa, fixing nitrogen only under anaerobic conditions. Anaerobic bacteria, typified by C.pasteurianum

generally are considered to use the thioclastic reaction (eq. 2) as a source of reducing power and energy for nitrogen fixation [27,92,93,95].

pyruvate + CoASH + 2 fd(ox) →

$$\text{acetylCoA} + CO_2 + 2\ \text{fd(red)} + 2\ H^+ \qquad \text{(eq.2)}$$

In the thioclastic reaction electrons are transferred from pyruvate to ferredoxin (fd) by pyruvate:ferredoxin oxidoreductase. Ferredoxin in turn reduces the Fe protein of nitrogenase. Pyruvate:ferredoxin oxidoreductases are thiamin diphosphate containing iron-sulphur proteins (M_r ca. 250000) [43]. In addition to pyruvate other substrates like H_2, formate, NADH and NADPH can support ferredoxin dependent nitrogenase activities in crude extracts from anaerobes [95]. But these reactions are thought of minor importance for electron transport to nitrogenase *in vivo* [30,92]. In anaerobes degradation of one molecule of pyruvate can give reduction of 2 molecules of ferredoxin and synthesis of 1 molecule of ATP via acetylCoA and acetylphosphate. Since nitrogenase needs at least 16 molecules of ATP against 8 molecules of reduced ferredoxin (eq. 1), it will be clear that not the production of reductant, but synthesis of ATP is limiting for nitrogenase activity in anaerobes.

Klebsiella pneumoniae is thus far the only nitrogen-fixer where the electron-transfer pathway to nitrogenase is genetically proven. In *K.pneumoniae* a (*nif*) gene cluster, comprising of 17 genes, is required for the *in vivo* synthesis and activity of nitrogenase. The products of *nif* F and *nif* J have been implicated in electron transport to nitrogenase [35,67,80]. *Nif* F^- and *nif* J^- mutant strains have no nitrogenase activity *in vivo* but have activity *in vitro* with dithionite. Crude extracts from wild type *K.pneumoniae* can couple the oxidation of pyruvate and formate to electron transport to nitrogenase [94], whereas extracts from *nif* F^- and *nif* J^- mutants cannot [35]. *Nif* J product was purified from wild type on the basis of its ability to restore nitrogenase activity to crude extracts of a *nif* J^- mutant when supplied with pyruvate as electron donor [76]. An earlier report on the purification of *nif* J [7] turned out to be questionable [76]. *Nif* F product was purified in a similar manner [61]. The *nif* F gene product was shown to be a flavodoxin of M_r 20000 ± 2000 [17,61,66]. The purified protein is a monomer and stable in O_2 [61]. *Nif* J

gene product was shown to be a pyruvate:flavodoxin oxidoreductase [76]. The enzyme catalyzes a thioclastic reaction (eq. 2) and is extremely sensitive to oxygen. The native protein (M_r 240,000) is an α_2 dimer and contains 8 moles of iron and 7 moles of acid-labile sulphide per mole of protein. Pyruvate is the only physiological substrate and only flavodoxin, not ferredoxin, is effective in coupling electron flow to nitrogenase. Spectral changes demonstrate that pyruvate:flavodoxin oxidoreductase reduces flavodoxin to the hydroquinone state which gets oxidized to the semiquinone by transferring electrons to nitrogenase. Without giving attention to the stoichiometry the components involved in electron transport to nitrogenase in Klebsiella pneumoniae can be placed in the next order:

$$\text{pyruvate} \rightarrow \underset{\text{oxidoreductase}}{\text{pyruvate:flavodoxin}} \rightarrow \underset{\underline{\text{nif}}\ \text{J}}{\text{flavodoxin}} \rightarrow \underset{\underline{\text{nif}}\ \text{F}}{Kp_2} \rightarrow Kp_1 \begin{cases} \rightarrow N_2 \\ \rightarrow NH_4^+ \end{cases} \qquad \text{(eq.3)}$$

Kp_1 and Kp_2 are the MoFe and Fe protein of nitrogenase.

1.4.2 Photosynthetic bacteria

This group includes the strict anaerobic sulphur bacteria Chlorobium and Chromatium and the facultatively aerobic non-sulphur bacteria Rhodopseudomonas and Rhodospirillum. The photosynthetic bacteria fix dinitrogen in the light under anaerobic conditions. Oxygen concentrations above 1 µM inhibit light-driven nitrogenase activity [29,37]. Only for the Chlorobiaceae it could be demonstrated that illuminated chromatophores generate reducing power for N_2-fixation [22]. For the other photosynthetic bacteria the redox potential of the primary electron acceptor is too high (-50 to -150 mV) to reduce ferredoxin directly. In the past six years evidence has been obtained for Rhodopseudomonas spp. [29,42,55,77], Rhodospirillum rubrum [54] and a member of the Chromatiaceae [41], that nitrogenase activity is not obligatory coupled to the activity of the pho-

tosynthetic apparatus. These organisms could fix nitrogen in the dark by aerobic respiration at low oxygen tensions. Depending on the organism, nitrogenase activity under dark aerobic conditions was 3-50% of the activities measured anaerobically in the light. After a period of adaptation, Rhodospirillum rubrum can even grow anaerobically in the dark [54,72]. Under these special conditions ferredoxin is reduced in the thioclastic reaction by pyruvate:ferredoxin oxidoreductase [26]. But it is unlikely that this enzyme is responsible for nitrogenase activity during photosynthetic or microaerophilic growth [27].

In 1980 it has been suggested by Haaker et al. [30] that the proton motive force is important for nitrogenase activity in photosynthetic bacteria. The proton motive force ($\Delta\tilde{\mu}_{H^+}$) is composed of a difference in proton concentration (ΔpH) and a difference in charge ($\Delta\psi$) across the cytoplasmic membrane. At 25°C:

$$\Delta\tilde{\mu}_{H^+} = \Delta\psi - 59\Delta pH \quad (mV) \qquad (eq.4)$$

According to the model [30] the energy present in the $\Delta\tilde{\mu}_{H^+}$ is used to lower the redox potential of electrons so that ferredoxin can be reduced. This process is called reversed electron flow. Indeed in 1982 it has been shown, that electron transport to nitrogenase in Rhodopseudomonas sphaeroides depends on a high membrane potential [29]. Furthermore the lower nitrogenase activity in the dark under microaerophilic conditions compared to the activity in the light under anaerobic conditions can be explained in terms of a lower membrane potential and a lower ATP/ADP ratio [29]. Evidence for the proteins, that are involved in this reversed electron flow might be obtained from analysis of nif$^-$ mutants defective in electron transport to nitrogenase, as isolated for Rhodopseudomonas capsulata [90].

1.4.3 Cyanobacteria (blue-green algae)

This group includes the unicellular Gloeothece and the filamentous algae like Plectonema boryanum and Anabaena cylindrica. In Anabaena species N_2-fixation takes place in specialized cells: the heterocysts. Heterocysts lack the oxygen evolving photosystem II and ribulose-1,5-bisphosphate carboxylase. It is thought that vegetative cells provide the heterocysts with carbon compounds (e.g. erythrose [65]) and the heterocysts return fixed nitrogen in the form of glutamine [81,91]. Cyanobacteria can fix nitrogen aerobically either in the light or in the dark. In the dark NADPH is thought to be the electron donor for nitrogenase, ferredoxin being directly reduced by ferredoxin:$NADP^+$ oxidoreductase [2,48]. In the light this pathway is switched-off. Electrons from NADPH first have to pass through photosystem I for ferredoxin reduction and thus light provides the reducing power for nitrogenase activity [74] (Fig. 3). In addition to NADPH also NADH and H_2 can donate electrons to the photosystem.

Serious objections against a linear electron transport chain from NADPH and via ferredoxin to nitrogenase were formulated by Haaker et al. [30]. The main objection being that in vivo the reducing power of NADPH is -350 mV [2], not low enough for N_2-fixation [74]. In vitro nitrogenase activity is measured with an NADPH regenerating system, which artificially increases the reducing power of NADPH. Haaker et al. [30] suggested a similar system to that of aerobic nitrogen-fixers: a proton motive force driven reversed electron flow to nitrogenase (see next section). In the light the proton motive force can be generated by cyclic electron flow, in the dark by respiration. Indeed experiments with intact organisms showed that the membrane potential was involved in sustaining nitrogenase activity both in heterocystous and non-heterocystous cyanobacteria [33,34]. Other authors demonstrated high nitrogenase activities in isolated heterocysts and cell-free extracts in the absence of energized membranes [38,74] and suggest that $\Delta\Psi$ is not the driving force for electron transport to nitrogenase, but some kind of a regulatory compound for N_2-fixation [9]. However nitrogenase activity without a $\Delta\Psi$ driven reversed electron flow would require a high

NADPH/$NADP^+$ ratio in the cell. Whereas it is clear that the enzymes involved in NADPH generation viz. glucose-6-phosphate- and isocitrate-dehydrogenase are negatively regulated by the reductant charge and ATP [2,62,69]. An explanation has been offered by proposing that a high NADPH/$NADP^+$ ratio is generated by light-dependent transhydrogenation of NADH. The NADH being reduced by glycolytic carbon degradation [73].

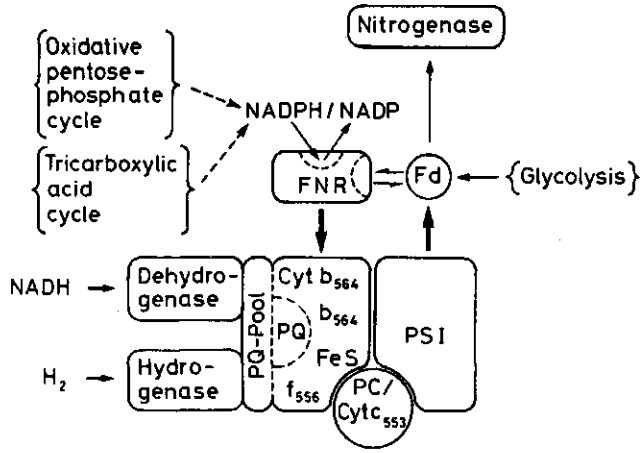

Fig. 3. Tentative scheme for electron flow from different sources to nitrogenase in heterocysts of Anabaena variabilis. Pathways supporting low nitrogenase activities are indicated by dashed arrows (involving dehydrogenases of the pentosephosphate pathway and tricarboxylic-acid cycle); high activities are indicated by solid arrows. NADH is formed by glycolytic degradation of glucose-6-phosphate, H_2 is formed by nitrogenase activity or may be supplied exogenously. Cyt, cytochrome; Fd, ferredoxin; FeS, Rieske iron-sulfur center; FNR, ferredoxin:NADP oxidoreductase; PC, plastocyanin; PQ, plastoquinone; PS I, Photosystem I. From ref. 74.

In the unicellular Gloeothece N_2-fixation and photosynthetic O_2 evolution occur in a single cell type. In nature cultures have photosynthesis in light and N_2-fixation in darkness [60]. But cells can also grow under constant illumination thereby evolving O_2 and fixing N_2 simultaneously. Ca^{2+} has been shown to be important in protecting nitrogenase from O_2

damage under these conditions [23]. Very recently it has been shown that respiration is the major source of reductant and ATP for nitrogenase activity both in the dark and in the light in Gloeothece, but photosystem I can contribute ATP at very high levels of illumination [56].

1.4.4 Obligate aerobic nitrogen-fixers

This group of bacteria includes the free living Azotobacter, Azospirillum species and micro-organisms like Rhizobium and Frankia spp., which can fix dinitrogen as free living organisms or in symbiosis with plants. One of the most puzzling observations for aerobic nitrogen-fixers is, that nitrogenase activity can be measured easily with dithionite in crude extracts, but no activity is observed with all kinds of physiological reductants. Thus far only for Azotobacter vinelandii, nitrogenase activity could be demonstrated in crude extracts using endogenous proteins and physiological substrates [5]. An electron transport pathway from NADPH to nitrogenase was proposed.

$$\text{NADPH} \xrightarrow[\text{oxidoreductase}]{} \text{ferredoxin} \xrightarrow[\text{unknown factor}]{} \text{flavodoxin} \rightarrow \text{nitrogenase} \qquad \text{(eq.5)}$$

The low redox potential necessary for electron flow to nitrogenase should be generated by a high NADPH/$NADP^+$ ratio [3]. As critisized by Haaker et al. [27,30] the proposed model (eq. 5) is thought to be too simplistic. Firstly, when eq. 5 is correct it must be possible to detect a reasonable nitrogenase activity with an NADPH generating system in crude extracts. For some Azotobacter strains the reaction rate was less than 5% of the activity with dithionite as electron donor but for other strains (including our strain A.vinelandii ATCC 478) no activity at all was observed. Secondly, the enzyme ferredoxin:$NADP^+$ oxidoreductase has not been found in Azotobacter spp. Thirdly, for nitrogenase activity the ratio NADPH/$NADP^+$ in A.vinelandii cells under nitrogen-fixing conditions is

approximately 0.4 [89] corresponding to a redox potential of -330 mV and therefore not low enough for nitrogenase activity.

An important observation was made in 1974 when it was shown that an energized cytoplasmic membrane is obligatory for electron transport to nitrogenase [28]. Later this observation was extended to bacteroids of Rhizobium leguminosarum [45] . After the discovery of a membrane-bound NADH-flavodoxin oxidoreductase [31] a scheme for electron transfer to nitrogenase of A.vinelandii was proposed (Fig. 4).

A membrane bound flavodoxin-oxidoreductase is reduced by NADH. The enzyme reduces flavodoxin semiquinone to the hydroquinone form by means of proton transport across an energized membrane. The flavodoxin hydroquinone

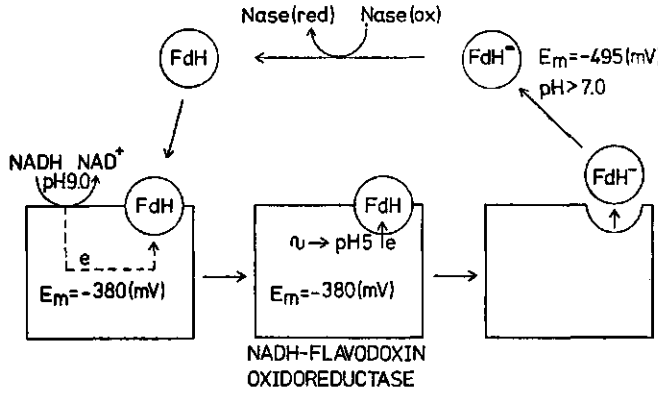

Fig. 4. Scheme for electron transfer to nitrogenase in Azotobacter vinelandii. The boxes represent an 'energised' membrane. From Haaker & Veeger [31].

transfers the electron to nitrogenase. For such a proton motive force driven reversed electron flow especially the membrane potential (eq. 4) turned out to be an important factor. This was shown both for A.vinelandii and Rhizobium leguminosarum [46,47]. Some support for a scheme like Fig. 4 was given by Howard et al. [39], who showed that although nitrogenase is a soluble enzyme [31,68] in the cell a structural association may exist between nitrogenase and the cytoplasmic membrane. Thus far attempts to induce

nitrogenase activity *in vitro* with NAD(P)H, flavodoxin and artificially energized membranes have been unsuccessful [87 and Haaker, unpublished results]. At the moment there is no definite proof, whether the scheme of Benemann (eq. 5) or Haaker (Fig. 4) or a combination of both is correct.

1.5 Outline of this thesis

The research reported in this thesis was focussed on the electron transport pathway to nitrogenase in *Azotobacter vinelandii*. As mentioned earlier the generation of reducing equivalents for nitrogenase is dependent on a high membrane potential. At least this was concluded from the short-term inhibitory effect of ammonium chloride on nitrogen fixation by *A.vinelandii* [47]. Later it was demonstrated by others that ammonia was no short-term inhibitor of nitrogenase activity in growing cultures [25]. In chapter 2 the discrepancies in the literature for the inhibition of electron transport to nitrogenase by NH_4Cl will be explained.

A detailed kinetic model for nitrogenase catalysis has been developed by Thorneley and Lowe [51-53, 82-85]. Their model predicts that nitrogenase catalysis is inhibited at high concentrations of the nitrogenase proteins, when the rate of reduction of oxidized Fe protein is not indefinitely fast. However their experiments were performed in the presence of the artificial electron donor dithionite. Chapter 3 deals with the catalytic activity of nitrogenase in intact cells. Evidence will be presented that the concentration of nitrogenase is high in intact cells, but that due to a very effective electron donating system nitrogenase activity is not inhibited. The enzyme activity in whole cells is even higher than ever measured *in vitro*.

Azotobacter vinelandii contains several low potential electron carriers, capable of donating electrons to nitrogenase [4,96,97]. At least two ferredoxins [96] and one flavodoxin [36], have been described to be present. As reviewed by Scherings [70] flavodoxin is the more likely candidate to serve as ultimate reductant for nitrogenase *in vivo*. But others are reluctant to accept this view [10]. Chapter 4 describes the isolation of three different flavodoxins from *A.vinelandii*, one being involved in

electron transport to nitrogenase. Proteins, that might probably play a role in the reduction of flavodoxin are described in Chapter 5. However the possibility is discussed that the pathway of flavodoxin reduction is not specific for nitrogen fixation. Evidence will be presented, suggesting that a direct interaction between electron transport to nitrogenase and electron flow through the respiratory chain may occur.

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2. INHIBITION OF NITROGENASE ACTIVITY BY AMMONIUM CHLORIDE IN *AZOTOBACTER VINELANDII*

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In *Azotobacter vinelandii* cells, the short-term inhibition of nitrogenase activity by NH_4Cl was found to depend on several factors. The first factor is the dissolved oxygen concentration during the assay of nitrogenase. When cells are incubated with low concentrations of oxygen, nitrogenase activity is low and ammonia inhibits strongly. With more oxygen, nitrogenase activity increases. Cells incubated with an optimum amount of oxygen have maximum nitrogenase activity, and the extent of inhibition by ammonia is small. With higher amounts of oxygen, the nitrogenase activity of the cells is decreased and strongly inhibited by ammonia. The second factor found to be important for the inhibition of nitrogenase activity by NH_4Cl was the pH of the medium. At a low pH, NH_4^+ inhibits more strongly than at a higher pH. The third factor that influenced the extent of ammonia inhibition was the respiration rate of the cells. When cells are grown with excess oxygen, the respiration rate of the cells is high and inhibition of nitrogenase activity by ammonia is small. Cells grown under oxygen-limited conditions have a low respiration rate and NH_4Cl inhibition of nitrogenase activity is strong. Our results explain the contradictory reports described in the literature for the NH_4Cl inhibition of nitrogenase in *A. vinelandii*.

In all free-living nitrogen-fixing bacteria examined thus far, synthesis of nitrogenase is repressed after addition of ammonia to the culture medium (2, 4, 11, 13, 15, 17, 19, 21). In most of the bacteria, except some photosynthetic bacteria, the nitrogenase proteins when measured in cell extracts remain active. Due to dilution and degradation, no nitrogenase activity is detected in cell extracts after several generation times (2, 4, 11, 17, 19).

In addition to this long-term effect of ammonia, with added ammonia some bacteria are capable of rapidly switching off whole-cell nitrogenase activity. Nitrogenase activity is inhibited within minutes after addition of NH_4^+ to whole cells. This so-called short-term effect is observed in *Azotobacter* (5), *Rhodospirillum* (13), *Rhodopseudomonas* (8, 21) and *Anabaena* (20) species. For the phototrophic organisms, inhibition is dependent on the growth conditions (1, 18, 20). In organisms that fix nitrogen only under fermentative conditions, like *Clostridium* and *Klebsiella* species, no short-term effect of ammonia is found (2, 19). For bacteroids of *Rhizobium leguminosarum*, it has been shown that ammonia is not taken up but excreted. Consequently, no inhibition of nitrogenase is found (12).

In a review Eady (5) noted that the extent of inhibition of nitrogenase activity by NH_4Cl in *Azotobacter vinelandii* is variable. Depending on the authors, the inhibition varied between 30 and 100% (4, 9, 11, 12). Later Gordon et al. (6) demonstrated that there is no short-term inhibition by ammonia of nitrogenase activity in growing cultures of *A. vinelandii*.

The results described in this paper resolve the discrepancies as to the extent of inhibition of whole-cell nitrogenase activity in *A. vinelandii* by NH_4Cl. Conditions will be defined under which nitrogenase activity in *A. vinelandii* is hardly inhibited by addition of NH_4Cl and also those where whole-cell nitrogenase activity is completely inhibited by addition of NH_4Cl.

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MATERIALS AND METHODS

Growth conditions of bacteria. *A. vinelandii* ATCC strain 478 (AV-478) was cultured on a Burk nitrogen-free basic salt medium (14) and supplied with sucrose as the sole carbon and energy source. Cells were grown in 0.5-liter batches in 2-liter Erlenmeyer flasks shaken at 300 rpm, in a New Brunswick controlled environment incubator shaker at 30°C. After growth of the culture, the optical density was measured at 660 nm. An optical density at 660 nm of 1.0 corresponded to 0.14 mg of bacterial protein per ml. A mutant of *A. vinelandii* OP (AV-11) was provided by D. V. DerVartanian. The respiratory chain of AV-11 appears to be blocked between cytochromes c_4 and c_5 and the oxidases o and a_1 (10). Cells were grown in the same way as was AV-478.

Measurement of nitrogenase activity of whole cells. *A. vinelandii* cells were grown as indicated. Cells were harvested by centrifugation for 10 min at 10,000 × *g*, washed once with distilled water, and suspended in Burk medium to a protein concentration of 30 mg/ml. No differences were observed between washed cells and growing cultures with respect to respiration rate, nitrogenase activity, and the effect of NH_4Cl. Cells were stored at 0°C during the course of the experiments. Nitrogenase activity of whole cells was measured in the system described by Haaker et al. (7). Cells were incubated in 5 ml of Burk medium in a thermostated vessel at 30°C. A closed gas phase consisting of air with 10% acetylene was pumped with an adjustable air pump through the incubation mixture. The free oxygen concentration in the medium was measured with a standard oxygen electrode. The detection limit of the O_2 electrode was 1 μM O_2. The oxygen input rate was calibrated as described earlier (7). Nitrogenase activity was measured as acetylene reduction by analyzing samples taken from the gas phase at suitable time intervals. By varying the gas flow through the cell suspension, whole-cell nitrogenase activity was measured at different oxygen input rates.

pH studies. Strain AV-478 was grown to a density of 0.5

mg/ml. Cells were harvested by centrifugation, washed once, and suspended in water to a protein concentration of 45 mg/ml. Cells (1.3 mg of protein per ml of incubation mixture) were incubated in the medium containing 25 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid-NaOH, 2 mM $MgCl_2$, 10 mM KCl, and 50 mM sucrose, at the pH as indicated. Cells were equilibrated for 8 min at an oxygen input of 0.9 μmol of $O_2 \cdot min^{-1} \cdot mg^{-1}$. The whole-cell acetylene reduction rate was measured shortly before and after addition of 28 mM NH_4Cl. The protein concentration of a cell suspension was determined by the method of Sedmak and Grossberg (16) after 1 min of sonication of the cell suspension. Bovine serum albumin (Sigma Chemical Co.) was used as standard.

Materials. Acetylene was purchased from Hoekloos (Amsterdam). Chemicals were of the highest analytical grade and were obtained from commercial sources.

RESULTS

Oxygen dependence of nitrogenase activity. The nitrogenase activity of intact *A. vinelandii* cells depends on the amount of oxygen present during the assay (3, 7). This is shown in Fig. 1 for organisms harvested at an early stage of growth. When these cells were incubated at a low oxygen input rate, the nitrogenase activity, measured as acetylene reduction, was low (Fig. 1). Under these conditions, addition of NH_4Cl caused a strong inhibition of the nitrogenase activity. With more oxygen, the nitrogenase activity increased to a maximum at an oxygen input rate of 1.7 μmol of $O_2 \cdot min^{-1} \cdot mg^{-1}$, when dissolved O_2 was almost 10 μM. Addition of NH_4Cl resulted in only 7% inhibition (Fig. 1). When the oxygen input rate was further increased, the nitrogenase activity was not inhibited until the free oxygen concentration exceeded 40 μM (Fig. 1). Under these incubation conditions, the inhibition of nitrogenase activity by NH_4Cl was strong. At an oxygen input of 3.5 μmol of $O_2 \cdot min^{-1} \cdot mg^{-1}$, addition of NH_4Cl inhibited nitrogenase activity by 95% (Fig. 1). From the results it is clear that the percentage of inhibition by NH_4Cl is variable. The inhibition is strong when whole cells are incubated at a low or a high oxygen input rate. At an optimum oxygen input rate for maximum nitrogenase activity, NH_4Cl is only slightly inhibitory.

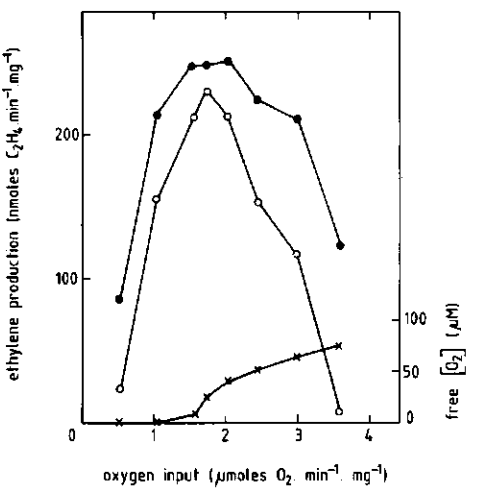

FIG. 1. Effect of the oxygen input rate on the inhibition of nitrogenase activity by NH_4Cl in cells grown with excess oxygen. Strain AV-478 was grown to a density of 0.09 mg of protein per ml and harvested. In an assay, cells (4 mg of protein) were preincubated for 5 min at an oxygen input of 1.6 μmol of $O_2 \cdot min^{-1} \cdot mg^{-1}$. After that time nitrogenase activity was measured for 10 min at the indicated oxygen input. Then, 28 mM NH_4Cl was added, and nitrogenase activity was measured for at least 10 min. Symbols: ●, activity without NH_4Cl; ○, activity in the presence of NH_4Cl; X, free oxygen concentration in the medium at the moment NH_4Cl was added.

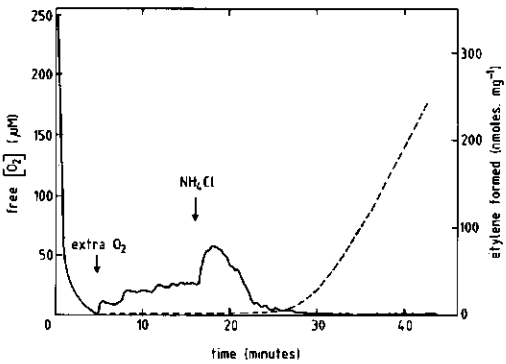

FIG. 2. Effect of NH_4Cl on the free oxygen concentration in the medium and on the nitrogenase activity during a whole-cell nitrogenase activity measurement. Strain AV-478 was grown to a density of 0.5 mg of protein per ml and harvested. At t = 0 min, cells (4.5 mg of protein) were added and preincubated at an oxygen input of 1.1 μmol of $O_2 \cdot min^{-1} \cdot mg^{-1}$. At t = 5 min, acetylene was added and the oxygen input was set at 1.3 μmol of $O_2 \cdot min^{-1} \cdot mg^{-1}$. At t = 16 min, 28 mM NH_4Cl was added. The solid line shows the oxygen concentration detected in the medium. The dashed line shows the total amount of ethylene that was formed.

When a culture was grown to a greater density and these cells were assayed for nitrogenase activity, a curve similar to that shown in Fig. 1 was obtained. However, due to a lower respiration rate of the cells, no nitrogenase activity could be detected as soon as free oxygen was detectable in the medium. It was also found that the inhibition of nitrogenase activity by NH_4Cl was much stronger. But under typical conditions it was possible to demonstrate a stimulation of the nitrogenase activity by the addition of NH_4Cl (Fig. 2). At t = 0 min, cells were added to the incubation mixture. The oxygen input rate was set to a value of 1.1 μmol of $O_2 \cdot min^{-1} \cdot mg^{-1}$. The cells respired at a rate such that the free oxygen concentration decreased to zero. At t = 5 min no free oxygen was detectable in the medium, and nitrogenase activity was observed (data not shown). When the oxygen input rate was increased to a value of 1.3 μmol of $O_2 \cdot min^{-1} \cdot mg^{-1}$ (extra O_2), free oxygen was detectable in the medium and nitrogenase activity was switched off. At t = 16 min NH_4Cl was added to the medium. After a temporary increase, the free oxygen concentration decreased to zero. When the free oxygen concentration was below 5 μM, nitrogenase activity was observed.

The apparent stimulation by NH_4Cl of nitrogenase activity is an indirect effect. Addition of NH_4Cl results in an increased respiration rate of the cells, and the nitrogenase activity of the cells is no longer switched off by the presence of free oxygen.

Effect of NH_4Cl concentration. The effect of the NH_4Cl concentration on the nitrogenase activity of whole cells was studied. In a concentration range of 0.35 to 56 mM NH_4Cl, no difference in the extent of inhibition was observed. At an initial concentration up to 1 mM NH_4Cl, inhibition was fully reversible during the experiments. This can be explained by assuming that all added ammonia was assimilated and nitrogenase activity returned to its original value.

Effect of pH. The pH of the incubation medium is an important factor for the extent of NH_4Cl inhibition (Table 1). In the absence of NH_4Cl, the nitrogenase activity of whole cells did not vary significantly with the pH value of the medium, but the inhibition of nitrogenase activity by NH_4Cl was found to be pH dependent. At pH 6.5, the acetylene reduction rate was strongly inhibited by addition of NH_4Cl. When the pH of the incubation medium was higher, inhibition by NH_4Cl was less (Table 1). At pH 7.5 and pH 8.0, the medium acidified rapidly after addition of NH_4Cl, and consequently the inhibition of nitrogenase activity increased with time.

Inhibition of nitrogenase activity by NH_4Cl at different stages of growth. A growth curve of *A. vinelandii* ATCC strain 478 is shown in Fig. 3. The cells were grown in an Erlenmeyer flask shaken at a constant rate. At the indicated times, samples were taken from the culture and the optical density, protein concentration, nitrogenase activity, and respiration rate of the cells were measured. The nitrogenase activity of the cells was measured at different oxygen input rates (Fig. 1). The maximum nitrogenase activity and the inhibition of the maximum nitrogenase activity by NH_4Cl were dependent upon the growth stage (Fig. 3). Up to 6.5 h after inoculation, little inhibition of the nitrogenase activity by NH_4Cl was observed. When the culture continued to grow, inhibition of nitrogenase activity by NH_4Cl increased. After 24 h of growth, the nitrogenase activity of the culture was much lower, and addition of NH_4Cl resulted in a 70% inhibition of the nitrogenase reaction when measured under optimal conditions. From these results it is clear that the extent of inhibition of nitrogenase activity by NH_4Cl depends on the stage of growth. At the end of growth, nitrogenase activity is more strongly inhibited than at an early phase of growth.

We also measured the respiration rate of the cells. It was observed that during growth the respiration rate of the cells (per milligram of protein) decreased with time. This is not surprising, since the oxygen input rate into the culture was constant while the protein content increased. Apparently the respiration rate of the cells adapted to lower amounts of oxygen available per cell. In samples taken from the culture during the first hours of growth, the cells had a high respiration rate. In these cells the nitrogenase reaction was not inhibited when free oxygen was present at detectable concentrations in the incubation medium (see also Fig. 1). In samples taken from the culture at later than 6.5 h of growth, nitrogenase activity was completely inhibited when free oxygen was detected in the medium. The inhibition of nitrogenase activity by NH_4Cl also increased (Fig. 3). So it seems that in cells with a high respiration rate, nitrogenase activity is relatively insensitive to free oxygen and to ammonia. However, in cells with a lower respiration rate, nitrogenase activity is sensitive to free oxygen and also to added NH_4Cl. This phenomenon was even more clearly observed with *A. vinelandii* OP mutant strain AV-11 (data not shown). When AV-11 was grown to a density such that free oxygen inhibited nitrogenase activity, inhibition by NH_4Cl was 100%.

TABLE 1. Effect of pH on the inhibition of nitrogenase activity by NH_4Cl

Initial pH	Ethylene production (nmol · min^{-1} · mg^{-1}):		Inhibition by NH_4Cl (%)
	$-NH_4Cl$	$+NH_4Cl$	
6.5	78	17	78
7.0	83	28	67
7.5	88	50	43
8.0	78	59	24

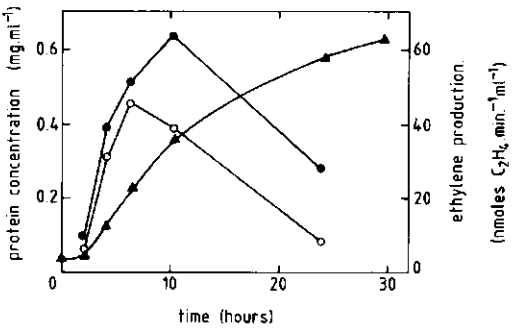

FIG. 3. Growth curve of *A. vinelandii* 478. AV-478 was grown in batch culture to a concentration of 0.3 mg of protein per ml as described in the text and diluted with fresh medium to a concentration of 0.036 mg of protein per ml at t = 0 h. At the times indicated, samples were taken and assayed as described in the text. Symbols: ▲, protein concentration; ●, maximum nitrogenase activity; ○, nitrogenase activity in the presence of 28 mM NH_4Cl.

DISCUSSION

Conflicting reports have appeared in the literature concerning the inhibition of nitrogenase activity by added NH_4^+ in whole cells of *Azotobacter* species (4–6, 9, 11, 12). Reports of the extent of the short-term inhibition of nitrogenase activity by NH_4^+ varied between 15 and 100%. In this report we show five reasons why this variation can be ascribed to differences in growth and test conditions. First, inhibition by NH_4Cl is dependent on the oxygen input rate during the test of whole-cell nitrogenase activity. Under conditions where nitrogenase activity is maximal, the inhibition by NH_4Cl is minimal (Fig. 1). Second, inhibition by NH_4Cl is dependent on the pH of the incubation mixture (Table 1). At a low pH, NH_4^+ inhibits more effectively than at a higher pH. Third, inhibition by NH_4Cl is dependent on the growth stage of cells (Fig. 3). In a culture growing rapidly, cells have a high respiration rate, and the nitrogenase activity is high and only slightly inhibited by addition of NH_4Cl. During oxygen-limited growth, the cells have a low respiration rate, and NH_4Cl is a strong inhibitor of nitrogenase activity. Fourth, inhibition by NH_4Cl is dependent on the carbon source used for growth. In succinate-grown cells inhibition by NH_4^+ addition is stronger than in sucrose-grown cells (12). Fifth, inhibition by NH_4Cl is only found when nitrogenase activity is measured in intact cells. No short-term effect is observed when the nitrogenase activity is measured in cell extracts (17). Gordon et al. (6), for instance, found no significant inhibition of nitrogenase activity by NH_4^+. Their experiments were done with growing cultures with high nitrogenase activities. We have shown (first and

third reasons) that inhibition by NH_4Cl under these conditions is minimal.

On the contrary Laane et al. (12) found strong inhibition of nitrogenase activity by addition of NH_4Cl. In their experiments the high concentrations of protein necessary for flow dialysis experiments were used. Under these conditions aeration of the cells is not optimal, and inhibition by NH_4Cl is expected to be strong (see first reason above). Furthermore, these authors harvested cells at the end of logarithmic growth, and we have shown that strong inhibition by NH_4Cl can be expected late in growth (see third reason above). We have also shown that it is possible to create conditions in which addition of NH_4Cl results in a stimulation of nitrogenase activity (Fig. 2). However, this can be explained by an effect of NH_4Cl on the respiration rate of the cells, thus relieving oxygen inhibition.

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3. THE CATALYTIC ACTIVITY OF NITROGENASE IN INTACT *AZOTOBACTER VINELANDII* CELLS

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The influence of the growth conditions on the concentration of nitrogenase and on the nitrogenase activity, was studied in intact *Azotobacter vinelandii* cells. It was observed that whole cell nitrogenase activity could be enhanced in two ways.

An increase of the growth rate of cells was accompanied by an increase in whole cell nitrogenase activity and by an increase in the concentration of nitrogenase in the cells. The molar ratio of Fe protein:MoFe protein was 1.47 ± 0.17 and independent of the growth rate. Activity measurements in cell extracts showed that the catalytic activity of the nitrogenase proteins was independent of the growth rate of cells.

The second way to increase whole cell nitrogenase activity was to expose cells to excess oxygen. Whole cells were exposed for 2.5 h to an enhanced oxygen-input rate. After this incubation nitrogenase activity was increased without an increase in protein concentration. It is calculated that the catalytic activity of the Fe protein in these cells was 6200 nmol C_2H_4 formed $\cdot$ min^{-1} $\cdot$ (mg Fe protein)$^{-1}$. With these cells and with cells grown at a high growth rate, 50% of the whole cell activity is lost by preparing a cell-free extract. It will be demonstrated that this inactivation is partly caused by the activity measurements *in vitro*. When dithionite was replaced by flavodoxin as electron donor, a maximal catalytic activity of 4500 nmol C_2H_4 formed $\cdot$ min^{-1} $\cdot$ (mg Fe protein)$^{-1}$ was measured *in vitro* for the Fe protein.

The results are discussed in relation to the present model for nitrogenase catalysis.

Nitrogenase is an enzyme system, that is capable of reducing atmospheric N_2 to ammonia. For catalysis an anaerobic environment, MgATP and a low-potential electron donor are obligatory. Nitrogenase is composed of two dissociating protein components: a tetrameric MoFe protein carrying the substrate-reducing site and a dimeric Fe protein. The properties of nitrogenases from different bacterial sources have been reviewed by Mortenson and Thorneley [1]. Until now it has been generally accepted that the Fe protein of any nitrogenase complex has one [4Fe-4S] cluster [1, 2]. However, in contrast to earlier data, Braaksma et al. [3, 4] reported that Fe protein can be isolated from *Azotobacter vinelandii* (Av_2) containing more than 4 Fe and $4S^{2-}$ atoms/molecule Av_2. The iron and sulphide contents of the protein were dependent upon the specific activity of the purified protein.

We developed a method to measure the catalytic activity of nitrogenase *in vivo*. Whole cell nitrogenase activity was measured with the acetylene reduction method and the amount of the nitrogenase proteins detected by protein blotting, a relatively new immunological method [5]. With these data the catalytic activities of Av_1 and Av_2 *in vivo* could be calculated. Cells were grown under different conditions with different whole cell nitrogenase activities. These cells were used to investigate whether the catalytic activity of nitrogenase is constant or depends upon the growth conditions of a culture.

Abbreviations. The MoFe and Fe proteins of nitrogenase of *Azotobacter vinelandii* are referred to as Av_1 and Av_2; Fld, flavodoxin; Fe/S II, iron-sulfur protein II [13]; SDS, sodium dodecyl sulfate; Tes, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]-amino}-ethanesulfonic acid.

Enzyme. Nitrogenase (EC 1.18.2.1).

In addition to nitrogenase the concentration of two other proteins, known to play a role in the physiology of nitrogen fixation, were determined. Flavodoxin, which is thought to be the physiological electron donor to nitrogenase [6–8] and Fe/S protein II, which protects nitrogenase from being inactivated by O_2 [9–11]. The relation between growth conditions, nitrogen fixation and the concentration of both proteins was studied.

A model for nitrogenase catalysis, developed by Thorneley and Lowe [12], predicts that nitrogenase will be inhibited at high protein concentrations. In this paper we show that the concentration of nitrogenase is high in *A. vinelandii* and not inhibited as observed with the purified proteins. The significance of our findings in relation to the model for nitrogenase catalysis will be discussed.

MATERIALS AND METHODS

Growth of bacteria and enzyme purification

Azotobacter vinelandii ATCC 478 was cultured on a Burk nitrogen-free basic salt medium [14] in a New Brunswick chemostat (type C30). Sucrose was supplied as the sole carbon and energy source. N_2-fixing cells were grown O_2-limited at an oxygen input rate of 50 mmol $O_2 \cdot l^{-1} \cdot h^{-1}$ at various dilution rates. As a control, cells were grown in the presence of 28 mM NH_4Cl at an oxygen input rate of 50 mmol $O_2 \cdot l^{-1} \cdot h^{-1}$ and a dilution rate of 0.1 h^{-1}. The pH of the growth medium was kept at 7.0 with NaOH.

In some experiments N_2-fixing cells, grown at an oxygen input rate of 50 mmol $O_2 \cdot l^{-1} \cdot h^{-1}$ and a dilution rate of 0.1 h^{-1}, were exposed 2.5 h to an increased oxygen input rate

of 110 mmol $O_2 \cdot l^{-1} \cdot h^{-1}$ (O_2 shock). During this O_2 shock, nitrogenase activity was inhibited and no growth detectable. To prevent dilution, the supply of fresh medium was stopped. During the 2.5-h period, the respiration rate of the cells adapts to the increased O_2 input rate (see also [15]) and the free oxygen concentration in the medium decreases. After 2.5 h the free oxygen concentration was lowered to 25 μM and growth of the culture was observed. At that moment a sample was taken out of the growth vessel. These cells are referred to as 'oxygen-shocked'.

The nitrogenase proteins Av_1 and Av_2 were purified as described earlier [3]. Flavodoxin was isolated together with Av_2. During the last purification step of Av_2 (a Sephacryl S200 column) both proteins were separated. Flavodoxin was then purified by an $(NH_4)_2SO_4$ precipitation between 65% and 95% saturation. After precipitation 1 mM riboflavin 5′-phosphate was added and the protein was dialysed against 10 mM Hepes pH 7.5, 0.1 mg/ml dithiothreitol. Purified flavodoxin had a ratio $A_{272}/A_{450} = 5.2$. The Fe/S protein II was purified as described by Scherings et al. [8].

Preparation of antisera

Antisera against Av_1, Av_2, Fld and Fe/S II were raised separately in New Zealand white rabbits using the procedure described by Voordouw et al. [5]. Fld and Fe/S II were injected directly. Prior to use in immunization, Av_1 and Av_2 were made free of final trace impurities by preparative SDS/polyacrylamide gel electrophoresis followed by extraction of the protein bands from the gel.

Concentration determinations

A. vinelandii cells, grown in a chemostat, were concentrated by centrifugation and suspended in 50 mM Tes/NaOH pH 7.5, 4% (w/v) SDS, 1 mM EDTA, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 0.001% (w/v) bromophenol blue to a protein concentration of 3 mg/ml. Cells were lysed by boiling, followed by 1 min sonification in an MSE sonifier. Polyacrylamide gel electrophoresis was carried out according to Laemmli [16] as described elsewhere [17]. For optimal results 10 μg protein was applied on a slab gel containing 14% acrylamide, 0.09% methylene-bisacrylamide. After electrophoresis proteins were transferred to nitrocellulose filters by the protein blotting method of Bowen et al. [18]. The nitrocellulose sheets were incubated overnight with antisera against Av_1, Av_2, Fld and Fe/S II. Bound antibodies were detected by incubation with ^{125}I-labelled protein A and, after washing, autoradiography of the nitrocellulose sheets [5]. By using the autoradiogram, radioactive bands on the nitrocellulose were cut out and counted in 7 ml Instafluor (Packard) in a Hewlett Packard liquid scintillation spectrometer. Calibration curves were made with purified proteins, which were applied on the same gel as the samples. The slope of the calibration curve depends upon the number of antigenic determinants that is recognized by the antiserum. Thus the amount of blackening on a particular X-ray film is not always linearly related to the amount of protein present. For the Fe/S protein II, the amount of radioactivity was plotted against the amount of protein. For the other proteins the amount of radioactivity was plotted against $\log_{10}$ of the amount of protein. Linear calibration curves were obtained with 5–25 pmol of pure polypeptides. The calibration curves were used to estimate the amount of Av_1, Av_2, Fld and Fe/S II in the cell samples. From the amount of protein (in μg/mg total protein) the concentration in the cell was calculated by using an internal volume for *Azotobacter* cells of 5 μl/mg total protein [19] and an M_r for Av_1 of 220000 [20, 21], for Av_2 of 63000 [22], for Fld of 23000 [23], for Fe/S II of 26000 [11].

Measurement of nitrogenase activity

The nitrogenase activity of whole cells was measured as acetylene reduction in samples taken out of the growth vessel of the chemostat as described elsewhere [24]. To prepare an extract, 200 ml of the culture was centrifuged at $10000 \times g$ for 10 min. Cells were suspended in 50 mM Tes/NaOH, 5 mM $MgCl_2$ pH 7.5 to a protein concentration of 25 mg/ml. Cells were broken at 0°C and under argon by sonification for four 30-s periods in an MSE sonifier. The nitrogenase activity of the extract was measured as acetylene reduction at different protein concentrations in the presence of an ATP-regenerating system as described earlier [3, 8]. Either 20 mM $Na_2S_2O_4$ or 4 mM $Na_2S_2O_4$ plus 130 μM flavodoxin was used as electron donor [25]. The maximum activity of Av_1 was measured after addition of a 10–20-fold molar excess of purified Av_2.

In this paper nitrogenase activity is expressed either as specific activity or as catalytic activity. The specific activity is the nitrogenase activity measured per mg total protein. The catalytic activity of Av_1 (or Av_2) is the nitrogenase activity expressed per mg Av_1 (or Av_2) present in a preparation. The amount of nitrogenase present in a preparation is determined by protein blotting as described above.

Protein concentrations were estimated with the Lowry method [26].

Materials

Nitrocellulose sheets (BA 85) were obtained from Schleicher & Schüll (Dassel, FRG). ^{125}I-labelled protein A from *Staphylococcus aureus* was prepared as described earlier [5]. All chemicals were of the highest commercial grades.

RESULTS

Concentration determination

SDS gel electrophoresis of extracts of *Azotobacter vinelandii* cells, cultured under conditions as described in Materials and Methods, followed by staining the gel with Coomassie brilliant blue, gave the polypeptide pattern shown in Fig. 1A. The polypeptide patterns of an extract of cells grown with N_2-fixing and of cells grown on NH_4Cl are shown. By applying purified proteins on the same gel the positions of Av_1, Av_2, Fld and Fe/S II were identified (lane M). The positions of these proteins are indicated by dashes. The amount of protein applied in lane M was approximately equal to the amount of the proteins in cell extracts. In the extract of N_2-fixing cells (−) the two subunits of Av_1 are visible. But Av_2, Fld and Fe/S II are hardly distinguishable from the other proteins on a stained gel. Transfer of the proteins from the gel onto nitrocellulose (protein blotting) followed by detection with specific antisera and ^{125}I-protein A gave the autoradiogram shown in Fig. 1B. On the gel the same samples were applied as in Fig. 1A. Av_1, Av_2, Fld and Fe/S II can be easily detected now both in the mixture of pure proteins (M) as well as in cell extracts. Only around Av_1 are some other protein bands visible on the blot. As will be discussed later, this cross-reactivity of the antiserum had no influence on the

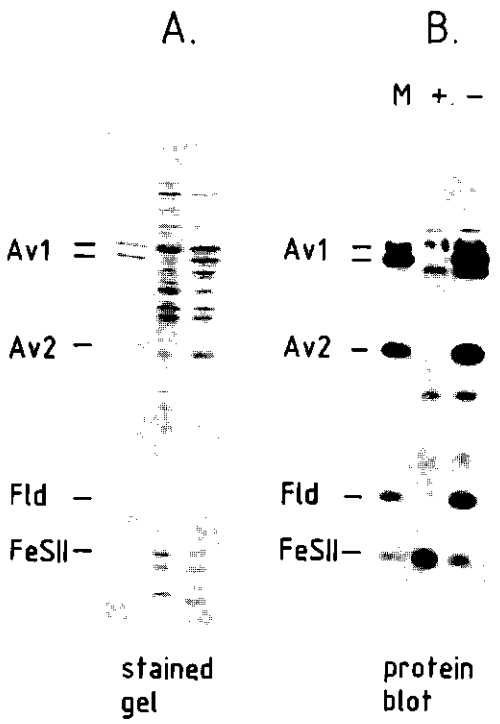

Fig. 1. *The presence of Av_1, Av_2, Fld and Fe/S II in cell extracts of* A. vinelandii. SDS gel electrophoresis was performed as described in Materials and Methods. (A) Gel after staining with Coomassie brilliant blue: (M) a mixture of purified Av_1 (2.12 μg), Av_2 (0.80 μg), Fld (0.51 μg), Fe/S II (0.13 μg); (+) 30 μg protein of an extract of cells grown in the presence of 28 mM NH_4Cl; (−) 30 μg protein of an extract of cells grown N_2-fixing. (B) Autoradiogram of a protein blot of the gel after treatment with antisera and ^{125}I-protein A: (M) a mixture of purified Av_1 (0.74 μg), Av_2 (0.28 μg), Fld (0.18 μg), Fe/S II (0.05 μg); (+) 10 μg protein of an extract of cells grown in the presence of 28 mM NH_4Cl; (−) 10 μg protein of an extract of cells grown N_2-fixing. The positions of Av_1, Av_2, Fld, Fe/S II after electrophoresis are indicated by dashes

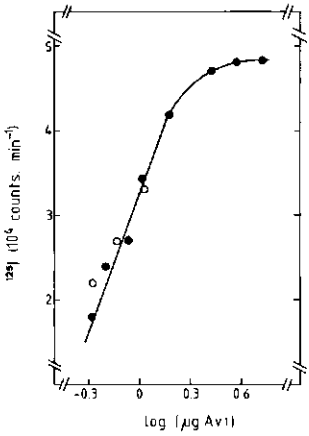

Fig. 2. *Calibration curve for Av_1.* Different amounts of purified Av_1 were applied on an SDS gel. After electrophoresis the gel was blotted onto a nitrocellulose sheet. The sheet was incubated with anti-Av_1 and ^{125}I-protein A as described in Materials and Methods. After autoradiography the bands originating from Av_1 were cut out and counted. The amount of radioactivity in the bands was plotted against the $\log_{10}$ of the amount of Av_1 applied on the gel (●——●). Purified Av_1 was mixed with 10 μg protein of an extract of cells grown on NH_4Cl (○)

determination of the concentration of Av_1. Furthermore the antiserum raised against Av_1 reacted more strongly with the α subunit compared to the reaction with the β subunit of Av_1. On most protein blots as well as on stained gels (Fig. 1 A) Av_2 appeared as two polypeptide chains with slightly different mobility. Due to over-exposure of the autoradiogram as shown in Fig. 1 B, Av_2 is only visible as one band. The phenomenon of two bands for the Fe protein is also observed in *Klebsiella pneumoniae* [27] and photosynthetic bacteria [28, 29]. But in contrast to the photosynthetic bacteria, there is no relation beween the presence or absence of these two bands and the activity of the Fe protein (not shown). Fig. 1 B shows that cells grown on NH_4Cl (+) have no detectable amounts of Av_1 and Av_2 and in comparison to N_2-fixing cells (−) contain less flavodoxin but more Fe/S protein II.

Using the autoradiogram (Fig. 1 B), the radioactive bands on the nitrocellulose sheets were cut out and the amount of radioactivity in each band was counted. On the gel different amounts of purified proteins were applied to construct a calibration curve. An example is given in Fig. 2. The amount of radioactivity was plotted against $\log_{10}$ of the amount of pure Av_1. In a range of 0.5 – 1.6 μg Av_1 a linear relationship was obtained between the radioactivity and the amount of pure protein applied. At higher concentrations of Av_1 (> 2.0 μg protein) the calibration curve flattened, possibly because of saturation of the nitrocellulose with protein. Also with extracts a linear relationship was found between the radioactivity at the spot of Av_1 and the amount of extract applied. When 10 μg protein of a cell extract was applied on a gel, the calibration curve of purified Av_1 could be used to estimate the concentration of Av_1 in the cell extract. When more protein was applied the amount of Av_1 present in the cell extract was under-estimated. With less extract the method was less accurate, because of a decreased binding of antibodies (and ^{125}I-protein A) compared to a constant background radioactivity. When 10 μg protein of a cell extract was used, the variation between duplicate determinations was less than 10%. As was mentioned earlier, the antiserum against Av_1 was cross-reactive with polypeptides with an almost similar M_r as Av_1 (Fig. 1 B). For quantification, Av_1 could be cut from the nitrocellulose filter without also taking the cross-reactive band with a slightly lower M_r. This was checked by autoradiography of the nitrocellulose filter after cutting out the Av_1 bands. In all cases the band with a slightly lower M_r was still present on the nitrocellulose paper. There is also a cross-reacting band with about the same M_r as the β subunit of Av_1. A typical experiment is shown in Fig. 1 B. The radioactivity on the spot of Av_1 was 26400 counts · min^{-1} for the N_2-fixing cells. The radioactivity of the cross-reacting material at the spot of Av_1 in cells grown on NH_4Cl was ony 3500 counts · min^{-1}. To test the influence of this cross-reactivity on the determination of Av_1, cell extracts of cells grown in the presence of NH_4Cl

Table 1. *Concentration of Av_1, Av_2, Fld and Fe/S II in intact* A. vinelandii *cells.*
Cells were grown under different conditions and the concentration of the proteins was determined as described in Materials and Methods. The mean values are given ± standard error of the mean for 4–7 separate experiments in each case of cells grown on nitrogen-free medium. The experiment with cells grown on NH_4Cl was carried out twice

Dilution rate	Special conditions	Density of the culture	Concentration of			
			Av_1	Av_2	Fld	Fe/S II
h^{-1}		mg protein · ml^{-1}	μM			
0.05	–	1.00 ± 0.05	40 ± 10	59 ± 10	220 ± 40	60 ± 40
0.10	–	0.80 ± 0.05	52 ± 5	74 ± 6	190 ± 25	62 ± 12
0.20	–	0.55 ± 0.05	62 ± 5	86 ± 4	220 ± 35	45 ± 15
0.25	–	0.45 ± 0.05	75 ± 5	111 ± 5	280 ± 30	40 ± 20
0.10	O_2 shock	0.85 ± 0.10	60 ± 10	90 ± 10	200 ± 20	50 ± 20
0.10	growth on NH_4Cl	1.20 ± 0.1	0	0	85 ± 20	90 ± 30

were mixed with purified Av_1. The effect on the calibration curve of the presence of extracts of cells grown on NH_4Cl was small (e.g. Fig. 2). Only for the lowest concentrations can it not be excluded that the amount of Av_1 is over-estimated for at most 20% due to cross-reactivity of the antiserum.

With the protein blotting method the amounts of Av_1, Av_2, Fld and Fe/S II were determined in *A. vinelandii* cells grown under different conditions (Table 1). When cells were growing at higher dilution rates the density of the culture decreased, whereas the concentrations of Av_1 and Av_2 in the cell increased. At a dilution rate of 0.25 h^{-1} the amounts of Av_1 and Av_2 per mass of total cell protein were nearly twice as much as the amounts in cells grown at a dilution rate of 0.05 h^{-1}. An explanation for the increase in enzyme concentration is that, in addition to O_2, the availability of fixed nitrogen became growth limiting. The concentration of flavodoxin was independent of the growth rate of cells while the concentration of Fe/S protein II decreased in cells growing at a higher dilution rate. However, the standard deviation in the determination of Fld and Fe/S II was significantly larger. Large differences were measured for these proteins between different cell preparations grown at the same dilution rate. The reason for this variation is unknown.

When cells grown oxygen-limited were exposed to an increased oxygen input rate for 2.5 h (O_2 shock) the respiration rate increased [15]. The O_2 shock gave a small increase in the concentrations of Av_1 and Av_2 compared to the situation before the shock. The concentration of flavodoxin remained constant, whereas the concentration of Fe/S protein II decreased somewhat (Table 1).

In all preparations of N_2-fixing cells tested the molar ratio Av_2:Av_1 was 1.47 ± 0.17 ($n = 30$). The concentration of Fe/S protein II was not directly related to that of nitrogenase. The ratio Fe/S II to Av_1 varied between 0.4 and 2.0.

Growth of bacteria in the presence of NH_4Cl gave denser cultures than cells grown N_2-fixing (Table 1). In NH_4^+-grown cells no Av_1 and Av_2 could be detected, but the Fe/S protein II was present in high concentrations. The concentration of flavodoxin was less than half its concentration in N_2-fixing cells.

Nitrogenase activity of cells and extracts

The nitrogenase activities of whole cells grown at different growth rates, as well as extracts prepared from these cells, were tested for nitrogenase activity (Table 2). When the growth rate of the cells increased (higher dilution rates), the nitrogenase activity of the cells increased (Table 2). A similar phenomenon is described by Post et al. [30] for cells growing with excess oxygen. Exposing cells to an O_2 shock led to a sharp increase in whole cell nitrogenase activity (Table 2). Jones et al. [15] showed that exposure to excess oxygen increases the respiration rate of *Azotobacter* cells. By performing the experiment in the presence of chloramphenicol these authors showed this adaptation of the respiratory chain to be only partially dependent on protein synthesis. With our culture, however, an O_2 shock in the presence of chloramphenicol gave a 90% loss of nitrogenase activity in whole cells as well as in extracts (Table 2). After the O_2 shock in the presence of chloramphenicol, the concentrations of the Av_1 and Av_2 proteins were reduced to 60% (not shown). Apparently in contrast to adaptation of respiration, protein synthesis is obligatory to retain nitrogenase activity during an O_2 shock. This is a remarkable observation because it is known that there is

Table 2. *Nitrogenase activity of whole cells and cell extracts*
A. vinelandii was grown under different conditions in a chemostat as described in Materials and Methods. The doubling time of the cells was calculated from the dilution rate of the chemostat. After measuring whole cell nitrogenase activity, an extract was prepared of each sample by sonication and the nitrogenase activity was measured with $Na_2S_2O_4$ and an ATP-regenerating system as described in Materials and Methods. A 10–20-fold molar excess of purified Av_2 was added to the extract to measure the maximum activity of Av_1. Nitrogenase activity is expressed as nmol C_2H_4 formed · min^{-1} · (mg total protein)$^{-1}$. The mean values are given ± standard error of the mean for 3–6 separate experiments in each case, except for the experiment with chloramphenicol which was carried out twice

Dilution rate	Special conditions	Doubling time	Specific nitrogenase activity of		
			whole cells	extract	extract + Av_2
h^{-1}		h	nmol · min^{-1} · mg^{-1}		
0.05	–	13.9	38 ± 2	45 ± 4	90 ± 5
0.10	–	6.9	69 ± 3	49 ± 5	95 ± 5
0.20	–	3.5	130 ± 5	70 ± 10	115 ± 10
0.25	–	2.8	158 ± 4	85 ± 5	155 ± 5
0.10	oxygen-shock	–	175 ± 3	91 ± 5	130 ± 10
0.10	oxygen-shock in the presence of 200 μg/ml chloramphenicol	–	8	8	15

no *de novo* synthesis of nitrogenase when cells are exposed to excess O_2 [31].

An increase in nitrogenase activity of whole cells can be the result of a better supply of reducing equivalents and/or ATP to nitrogenase, or it can be caused by an increase in the activity of the enzyme nitrogenase itself. To discriminate between these two possibilities the whole cell activity was compared to the activity of Av_1 and Av_2 in extracts (Table 2). Since in all preparations (whole cells and extracts) the molar ratio Av_2:Av_1 was 1.5, the activity of the preparations is the specific activity of Av_2. The specific activity of Av_1 in extracts was measured by adding excess purified Av_2. When the whole cell activity increased from 38 to 69 nmol C_2H_4 formed $\cdot$ min^{-1} $\cdot$ mg $protein^{-1}$ there was no significant increase in the nitrogenase activity of the extracts. At higher whole cell activities, the extract activity was somewhat higher, but not proportional to the increase in whole cell activity. The same phenomenon was observed with cells that were exposed to an excess of oxygen. This means that under these growth conditions whole cell nitrogenase activity is mainly determined by the electron transport to nitrogenase and/or ATP supply.

A problem with the results shown in Table 2 is that the nitrogenase activity of extracts is lower than the activity *in vivo*. This could be caused by (a) inactivation of Av_2, the most labile nitrogenase component, during the preparation of an extract; (b) the presence of an inhibitor of nitrogenase in a crude extract [32]; (c) $Na_2S_2O_4$ being an inferior electron donor in extracts compared to the physiological donor flavodoxin [7, 25]. The first explanation was excluded by varying the sonification time of an extract. No effect was observed on the specific activity of nitrogenase in a cell-free extract. Also, purified Av_2 was added to a cell preparation after which the mixture was sonificated in the absence of $Na_2S_2O_4$. Only 10% of the activity of added Av_2 was lost during sonification compared to a control experiment where purified Av_2 was added to a similar cell extract during the assay of nitrogenase activity. Explanation (b) was excluded by adding purified Av_1 and Av_2 in a ratio 1:1.5 to an extract. The measured activity was the sum of the activities of the extract and the pure proteins separately. Explanation (c) was tested by using reduced flavodoxin as electron donor for Av_2 [7, 8, 25]. It is known from the literature that flavodoxin increases the specific activity of purified Av_2 more than 50% relative to its activity with $Na_2S_2O_4$ as a reductant [25]. We tested flavodoxin as a reductant in several crude extracts and also observed an increase in nitrogenase activity of approximately 50%. For instance, in an extract prepared from cells after an O_2 shock the activity was 90 nmol C_2H_4 formed $\cdot$ min^{-1} $\cdot$ mg^{-1} when measured with $Na_2S_2O_4$ and 138 nmol C_2H_4 formed $\cdot$ min^{-1} $\cdot$ mg^{-1} when flavodoxin was added. With flavodoxin as electron donor no excess of purified Av_2 was necessary to measure the maximum activity of Av_1. The maximum activity of Av_1 itself was not influenced by flavodoxin. These results indicate that in a whole cell the nitrogenase activity is higher than in extracts, because flavodoxin is a more effective electron donor for Av_2 than $Na_2S_2O_4$ and due to this effect Av_2 is more active in nitrogenase catalysis. However, such a statement should be taken *cum grano salis*, since even with flavodoxin as electron donor whole cell activities like 175 nmol C_2H_4 formed $\cdot$ min^{-1} $\cdot$ mg^{-1} were never measured in crude extracts.

On the catalytic activities of Av_1 and Av_2

From the results presented in Table 1 (concentrations of Av_1 and Av_2) and Table 2 (the specific nitrogenase activities of the same preparations) the catalytic activities of Av_1 and Av_2 *in vivo* and *in vitro* were calculated (Table 3). The catalytic activity of Av_1 in extracts was 1900 nmol C_2H_4 formed $\cdot$ min^{-1} $\cdot$ mg Av_1^{-1} independently of the growth conditions of cells. *In vivo* this activity is observed in cells grown at a high dilution rate. In these cells Av_1 is fully active. Apparently the amount of Av_2 present in the cell is capable of reducing Av_1 at maximum rate, despite the low ratio of Av_2:Av_1 of 1.5.

Table 3. *Catalytic activities of Av_1 and Av_2*
The data of Table 1 and 2 were combined to calculate the catalytic activities of Av_1 and Av_2 in whole cells and in cell extracts. Nitrogenase activity is expressed as nmol C_2H_4 formed $\cdot$ min^{-1} $\cdot$ (mg protein)$^{-1}$

Dilution rate	Special conditions	Catalytic activity of Av_1		Av_2	
		in vivo	*in vitro*	*in vivo*	*in vitro*
h^{-1}		nmol $\cdot$ min^{-1} $\cdot$ mg Av_1^{-1}		nmol $\cdot$ min^{-1} $\cdot$ mg Av_2^{-1}	
0.05	–	900 ± 200	2000 ± 400	2000 ± 400	2400 ± 400
0.1	–	1200 ± 100	1700 ± 200	3000 ± 300	2100 ± 300
0.2	–	1900 ± 200	1700 ± 200	4800 ± 300	2600 ± 400
0.25	–	1900 ± 150	1900 ± 150	4500 ± 250	2400 ± 200
0.1	O_2 shock	2600 ± 400	2000 ± 200	6200 ± 800	3200 ± 400

The catalytic activity of Av_2 in extracts was 2400 nmol C_2H_4 formed $\cdot$ min^{-1} $\cdot$ mg Av_2^{-1} independently of the growth rate of cells. Only after an O_2 shock, the catalytic activity of Av_2 increased to 3200 nmol C_2H_4 formed $\cdot$ min^{-1} $\cdot$ mg Av_2^{-1}. These catalytic activities of Av_2 were measured *in vitro* with $Na_2S_2O_4$ as electron donor. With reduced flavodoxin the activities are approximately 50% higher (see above). For cells grown at a low dilution rate (D = 0.05 h^{-1}) the catalytic activity of Av_2 in the cells is lower than the activity *in vitro*. At a high dilution rate the catalytic activity of Av_2 *in vivo* is much higher than the activity *in vitro* (up to 6200 nmol C_2H_4 formed $\cdot$ min^{-1} $\cdot$ mg Av_2^{-1}).

DISCUSSION

In this paper we have shown that protein blotting [5, 18] can be used to determine the amounts of single proteins in complex mixtures. The method has the advantage that cells are lysed by boiling in SDS and 2-mercaptoethanol and that SDS electrophoresis is carried out as the first step. This reduces the chance of under-estimating the amount of a certain protein caused by incomplete lysis of cells, proteolysis or denaturation of protein. Another advantage is that the method is quantitative without the need of having completely monospecific antisera (see Fig. 1 B). A third advantage is that the concentrations of different proteins can be measured simultaneously in one sample. In our case this means that the ratio Av_1:Av_2:Fe/S II could be measured accurately in all samples. The main disadvantages are that the method is time-consuming and that calibration curves are linear only in a narrow range of protein concentrations (Fig. 2).

In N_2-fixing cells the concentration of Av_1 varies between 40 µM and 75 µM and that of Av_2 between 60 µM and 110 µM depending on the growth conditions. The faster the cells grow the more nitrogenase is present. The molar ratio

Av_2:Av_1 was 1.47 ± 0.17 ($n = 30$) and was independent of the growth rate. For *Azotobacter chroococcum* grown under oxygen-limited conditions a ratio Fe protein:MoFe protein of 2.0 – 3.3 is reported [32]. This ratio was estimated by using SDS/polyacrylamide gel electrophoresis, a method which in our hands gives inaccurate values (see Fig. 1A). In *Klebsiella pneumoniae* and *Rhizobium leguminosarum* bacteroids the concentration of the Fe protein is 4-fold and 2.5-fold higher, respectively than the concentration of the MoFe protein [33 – 35]. So in *Azotobacter vinelandii* the ratio Fe protein:MoFe protein is relatively low compared to *K. pneumoniae* and *R. leguminosarum* bacteroids. We think this is due to a more efficient electron-donating system to the Fe protein in *A. vinelandii*. Because both the concentration and the activity of nitrogenase were measured in the same preparations, the catalytic activities of Av_1 and Av_2 could be calculated in whole cells (activity *in vivo*) and in cell extracts (activity *in vitro*, see Table 3). An increase in growth rate of cells is accompanied by an increase in whole cell nitrogenase activity, but in extracts no differences are measured for the catalytic activities of Av_1 and Av_2. This means that nitrogenase with the same catalytic activity is present in all cultures. Only in the case of an O_2 shock is the catalytic activity of Av_2 higher. This protein is probably the Fe protein with enhanced Fe/S content, as described by Braaksma et al. [3, 4].

A higher catalytic activity was calculated for Av_2 *in vivo* than was measured in extracts with $Na_2S_2O_4$. The difference between whole cell and extract activity was smaller when reduced flavodoxin instead of $Na_2S_2O_4$ was used as electron donor. The results indicate that in the cell the electron-donating system to nitrogenase and the ATP supply are more effective than in an assay with $Na_2S_2O_4$ and an ATP-regenerating system. Another effect of the efficient electron supply to Av_2 *in vivo* is that, due to the enhanced activity of Av_2 in intact cells, the maximum activity of Av_1 is measured although the molar ratio Av_2:Av_1 is 1.5. With purified components, Av_1 is not fully active at a ratio Av_2:Av_1 of 1.5. Addition of excess Av_2 gives at least a fivefold stimulation of the activity [36, 37].

We can state conclusively that despite the low ratio Av_2:Av_1 and the high intracellular concentrations of these proteins, the catalytic activities of Av_1 and Av_2 *in vivo* are remarkably high. This observation seems to be in contrast to the model proposed by Thorneley and Lowe [12] in which the rate-limiting step in nitrogenase catalysis is the dissociation of oxidized Fe protein from reduced MoFe protein after MgATP-induced electron transfer between these proteins. Their model predicts that nitrogenase is inhibited at high protein concentrations due to the association reaction of oxidized Fe protein with MoFe protein. However, no inhibition of nitrogenase activity *in vivo* is observed in *A. vinelandii* (Table 2). One explanation for this observation is that, *in vivo*, the concentration of oxidized Av_2 is kept very low by an effective electron-donating system to nitrogenase. Another possibility is that in the situation *in vivo* dissociaton of oxidized Av_2 from Av_1 is unnecessary, because flavodoxin could reduce oxidized Av_2 that is still bound to Av_1. We think that kinetic experiments have to be performed at high protein concentrations and with flavodoxin as electron donor instead of $Na_2S_2O_4$ to solve the problem.

The concentrations of the other proteins, flavodoxin and Fe/S protein II, which are important for the nitrogenase activity *in vivo*, were measured. Flavodoxin is thought to be the physiological electron donor to nitrogenase in *Azotobacter* species [6 – 8], and Fe/S protein II is involved in oxygen protection [8 – 11].

For *A. chroococcum* coordinate synthesis of nitrogenase and flavodoxin was demonstrated [31]. However, for *A. vinelandii* it has been known for a long time that flavodoxin is also present in cells grown on ammonia [6]. We have shown that the concentration of flavodoxin is small in NH_4^+-grown cells compared to the concentration in N_2-fixing cells (Table 1). In a preliminary experiment we also observed that the electrophoretic behaviour of flavodoxin on two-dimensional gels is different for flavodoxin present in cells grown on NH_4^+ compared to cells grown N_2-fixing. Furthermore, synthesis of flavodoxin parallels the synthesis of Av_1 and Av_2 after derepression (Klugkist, unpublished). So at least part of the flavodoxin that is present in *A. vinelandii* seems to be *nif*-specific. In contrast, the Fe/S protein II is not *nif*-specific. The concentration of this protein is high in cells grown on NH_4^+ (Table 1) and, as for *A. chroococcum* [31], synthesis of this protein is not related to the synthesis of nitrogenase. This suggests that the Fe/S protein II may have additional functions to that of O_2 protection of nitrogenase. From experiments *in vitro* it is known that an oxygen-stable nitrogenase complex can be formed by mixing Av_1:Av_2:Fe/S II in a ratio 1:1:1 or 1:2:1 [11, 38, 39]. However, it cannot be excluded that other ratios also give an oxygen-stable complex [11]. In whole cells the ratio varies between 1:1.5:0.4 and 1:1.5:2.0. We did not find a reason for this variation but from our results one can deduce that there might be an inverse relation between Fe/S protein II concentration and the rate of respiration of the cells. When cells have a high respiration rate, nitrogen fixation is protected by the enhanced respiration and conformational protection by Fe/S protein II is less important. This explains the results of Post et al. [30] who report a loss of nitrogenase activity after giving a short oxygen pulse to *A. vinelandii* cells grown with excess oxygen.

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4. CHARACTERIZATION OF THREE DIFFERENT FLAVODOXINS FROM *AZOTOBACTER VINELANDII*

4.1 SUMMARY

The flavodoxins from Azotobacter vinelandii cells grown N_2-fixing and from cells grown on NH_4Ac have been purified and characterized. The purified flavodoxins from these cells are a mixture of three different flavodoxins (Fld I, II, III) with different primary structures. The three proteins are separated by fast protein liquid chromatography; Fld I is eluted at 0.38 M KCl, Fld II at 0.43 M KCl and Fld III at 0.45 KCl. The most striking difference between the three flavodoxins is the mid-point potential (pH 7.0, 25°C) of the semiquinone/hydroquinone couple, which is -320 mV for Fld I and -500 mV for the other two flavodoxins (Fld II and Fld III).

All three flavodoxins are present in cells grown on NH_4Ac. In cells grown on N_2 as N-source only Fld I and Fld II are found. The concentration of Fld II is 10-fold higher in N_2-fixing cells as in cells grown on NH_4Ac. Evidence has been obtained that Fld II is involved in electron transport to nitrogenase.

As will be discussed, our observation that preparations of Azotobacter flavodoxin are heterogeneous, has consequences for the published data.

4.2 INTRODUCTION

Flavodoxin from Azotobacter vinelandii was first isolated by Shethna et al. in 1964 [1,2]. Since then this FMN containing redox protein has been known by the names "Azotobacter free-radical flavoprotein" [3], "Azotoflavin" [4] and "Shethna flavoprotein" [5]. Van Lin and Bothe [6] classified the Shethna flavoprotein as a flavodoxin. Azotobacter flavodoxin differs from other flavodoxins that it is a constitutive component of the cell, whereas flavodoxins from other organisms are induced by iron deficiency [7].

The protein consists of a single polypeptide chain with 179 amino acid residues, it contains one FMN and has a relative molecular mass of 19990 [8]. There is one single cysteine residue present which can cause dimerization of two flavodoxin molecules, a process which results in the loss of biological activity [9]. In addition to the 5'phosphate ester on the FMN, flavodoxin contains 2 moles of tightly bound phosphor groups [10]. One phosphate group is covalently bound to the protein in a phosphodiester linkage between serine and threonine residues. It has been suggested that the other is an acid labile phosphate in an acyl phosphate linkage with a protein COOH group [11]. At pH 8.0 and 25°C the redox potential of the quinone/ semiquinone couple (E2) of flavodoxin is -250 mV [12-14]. However an anomalous value of +50 mV was also reported for E2 [15]. The redox potential of the semiquinone/hydroquinone couple (E1) is -500 mV [12-16].

The primary function of the flavodoxin in Azotobacter species was suggested to be electron transport to nitrogenase. In 1969 Benemann et al. [4,17] showed that flavodoxin was one of the four factors native to A.vinelandii cells needed for electron transport from NADPH to nitrogenase, however the reported rate was just a fraction of the activity obtained with dithionite as electron donor. It appeared that the endogenous enzyme system was not capable of reducing flavodoxin effectively beyond the semiquinone state, whereas the hydroquinone form is necessary for nitrogenase activity [18,19]. In fact completely reduced flavodoxin turned out to be a good electron donor for nitrogenase; activities being 50% higher than with dithionite [19-21]. Furthermore flavodoxin from Azotobacter chroococcum was shown to be nif specific [22].

What argues against flavodoxin being the unique physiological electron donor for nitrogenase in A.vinelandii is the observation that flavodoxin is also present in cells grown on ammonia [4]. Such cells do not fix nitrogen and have no nitrogenase. Therefore it does not seem logical that these cells contain flavodoxin. However, recently we have shown that the concentration of flavodoxin is small in NH_4^+-grown cells compared to its concentration in N_2-fixing cells [21]. It also seemed that different species of flavodoxin were synthesized in the two modes of growth. In this paper we show that three different flavodoxins can be

isolated from Azotobacter vinelandii ATCC 478. One of the three flavodoxins is specific for cells grown on ammonia, and one flavodoxin seems to be nif specific.

4.3 MATERIALS AND METHODS

Growth of bacteria

Azotobacter vinelandii ATCC 478 was cultured in a 30 l fermentor of Bioengineering (type LP 30) on Burk's basic salt medium [23] with sucrose as the sole carbon and energy source. Cells were grown under N_2-fixing conditions or in the presence of NH_4Ac. During growth on NH_4Ac the concentration of ammonia in the medium was measured [24] and kept at 28 mM. Cells were harvested during the logarithmic phase and stored at -80^oC.

Purification of flavodoxin

Flavodoxin from N_2-fixing cells was isolated as described earlier [21]. Flavodoxin from NH_4Ac grown cells was isolated aerobically at 4^oC in 50 mM Tris-Cl, 1 mM EDTA, 0.1 mg/ml dithiotreitol, 0.1 mM PMSF, final pH 7.5 unless indicated otherwise. Cells (350 g) were disrupted with a Manton Gaulin homogenizer at 8000 psi. Unbroken cells were removed by centrifugation. The supernatant was applied onto a DEAE-Sephacel column (2.5 x 14 cm). The column was washed with buffer and buffer + 0.1 M NaCl after which a gradient (200 ml) of 0.1-0.7 M NaCl was applied. Flavodoxin eluted at 0.3 M NaCl. The pooled flavodoxin fractions were dialysed against buffer and brought to 50% saturation with $(NH_4)_2SO_4$ at 0^oC. After 1 hour the mixture was centrifuged 15 min at 15,000 x g. The supernatant was applied to a DEAE-Sephacel column (2.5 x 5 cm) equilibrated with 50% $(NH_4)_2SO_4$. The

column was washed with 50% $(NH)_2SO_4$ and a decreasing gradient (150 ml) of 50% to 0% $(NH_4)_2SO_4$ in buffer was applied [25]. Flavodoxin containing fractions eluted at 25% $(NH_4)_2SO_4$, were concentrated by ultrafiltration using an Amicon YM 10 filter and further purified on a Sephacryl-S200 column (1 x 60 cm). The yellow coloured fractions were concentrated by ultrafiltration and stored at -80°C. The ratio $A_{274/452}$ was 4.85.

Separation of flavodoxins

The purified flavodoxins isolated from either ammonia grown or N_2-fixing cells were separated on a mono Q HR 5/5 anion exchanger (0.5 x 5 cm) using an FPLC apparatus of Pharmacia Fine Chemicals. The sample was applied in 20 mM 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol pH 6.4 and eluted with a linear gradient (25 ml) of 0.25-0.55 M KCl. The pH of the eluted fractions was adjusted to pH 7.5 and 1 mM EDTA plus 0.1 mg/ml dithiothreitol was added. After several runs, flavodoxin fractions were concentrated by ultrafiltration and stored at -80°C.

Spectroscopy

^{31}P-NMR spectra were recorded at 5°C on a Bruker CXP 300 NMR spectrometer operating at 121 MHz. Wilmad 10 mm precision NMR tubes were used. The spectrometer was locked on the deuterium resonance of the D_2O solvent (10%) in the sample. Broadband proton decoupling of 0.5 W was applied. All spectra were recorded with 30° pulses and a repetition time of 1.1 s. Chemical shifts were determined relative to an external standard of 85% phosphoric acid. As recommended by the International Union of Pure and Applied Chemistry downfield shifts were regarded as positive. This in contrast to earlier reports [10,11].

Optical spectra were scanned on an Aminco DW2A spectrophotometer. Extinction coefficients were determined as described by Mayhew and Massey [26], except that flavodoxin was photoreduced with 5-deazaflavin and tricine [19]. Protein concentrations were estimated with the microbiuret method with bovine serum albumin as a standard [27]. The oxidation/reduction potential (E1) for the semiquinone/hydroquinone couple of flavodoxin I was measured after equilibration with NADPH in the presence of ferredoxin-$NADP^+$ oxidoreductase [28]. For flavodoxins II and III, E1 of the semiquinone/hydroquinone couple was determined after equilibration with H_2 in the presence of hydrogenase [28].

Electrophoresis

SDS-polyacrylamide gel electrophoresis was carried out according to a modified Laemmli method [29,30]. Gels contained 14% or 20% acrylamide with 0.09% or 0.07% bisacrylamide respectively. Molecular mass markers used, were: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), bovine carbonic anhydrase (30 kDa), α-chymotrypsinogen (25.7 kDa), myokinase (21 kDa), soybean trypsin inhibitor (20 kDa), myoglobin (17.2 kDa), α-lactalbumin (14.4 kDa). Two dimensional gel electrophoresis was performed by the O'Farrell technique [31] except that Nonidet NP40 was replaced by Triton X-100. Ampholytes (Pharmacia) in the pH ranges 3-10 and 4-6.5 were mixed at a ratio of 1:4. Protein blotting and concentration determinations of the three flavodoxins in intact A.vinelandii cells was done as described earlier [21]. The procedure of Cleveland [32] was used for peptide mapping by limited proteolysis in solution with chymotrypsin A4 (Boehringer) and protease from Staphylococcus aereus V8 (Miles Laboratories).

Cross reactivity of antisera

Antisera against flavodoxin I, II (isolated from N_2-fixing cells) and flavodoxin III (from cells grown on NH_4Ac) were raised separately in New Zealand White rabbits [33].

An SDS-polyacrylamide gel was run with the following samples: Fld I (0.2 μg), Fld II (0.25 μg), Fld III (0.2 μg), and a sample without protein. After electrophoresis proteins were transferred to nitrocellulose filters (Schleicher & Schüll) [34]. One filter was incubated overnight with the antiserum raised against Fld I. Bound antibodies were detected by incubation with ^{125}I-labelled protein A. After washing, an autoradiogram was made from the nitrocellulose sheet [21]. The duplicate filter was incubated overnight with antiserum raised against Fld II and handled in the same way. Both autoradiograms were used to cut out Fld I, Fld II and Fld III from the nitrocellulose filters. The radioactivity bound to the different proteins was counted [21]. The same procedure was carried out for another gel, except that the filters were incubated with antisera raised against Fld II and Fld III.

Immunoprecipitation after derepression

A.vinelandii was grown in a batch culture on Burk's medium with sucrose and 28 mM NH_4Ac. After washing two times with one volume of nitrogen-free medium, cells (10 ml) were incubated at 30°C in Burk's nitrogen-free medium containing 50 μM SO_4^{2-} and sucrose [35]. The incubation was aerated to a free oxygen concentration of 2 μM. After 15 min 25 μM $Na_2{}^{35}SO_4$ (250 μCi, New England Nuclear) was added. At t=25 min nitrogenase activity could be detected, which activity increased until t=60 min. Then 50 mM Na_2SO_4 was added. 1 ml of the culture was centrifuged and the pellet was washed with 1 ml of 10 mM Tris-Cl, 10 mM EDTA, final pH 8.0 and boiled in 0.1 ml 50 mM Tris-Cl pH 8.0, 1 mM EDTA, 4% SDS. After addition of 1 ml RIA buffer (10 mM Tris-Cl pH 7.5, 0.15 M NaCl, 1% Triton X-100, 0.1% Na-deoxycholate, 0.5% BSA) the solution was centrifuged. The supernatant containing the solubilized proteins was incubated with 15 μl antiserum against Fld I and 15 μl anti-Fld II for 75 min at 37°C followed by 5 hours at 4°C. Then 5 μl goat-anti rabbit IgG was added and the mixture incubated overnight at 4°C. Immunocomplexes were precipitated by centrifugation. The pellet was

washed two times with RIA buffer and finally taken up in sample buffer for electrophoresis [30].

As a control the same experiment was carried out, but with 28 mM NH_4Ac present during the incubation of whole cells. These cells remained repressed for N_2-fixation.

Immunoprecipitates of repressed and derepressed cells were analyzed on 14% SDS-polyacrylamide gels, with purified Fld I and II as reference. After electrophoresis the gel was stained to detect the Fld I and II markers. ^{35}S-labelled proteins present in the immunoprecipitates were visualized by autoradiography.

Materials

DEAE-Sephacel, Sephacryl-S200; Mono Q HR 5/5 were from Pharmacia Fine Chemicals (Uppsala, Sweden). All other chemicals were of the highest commercial grades. Megasphaera elsdenii hydrogenase was a gift from Mrs. A. van Berkel-Arts. Spinach ferredoxin-$NADP^+$ oxidoreductase was a gift from Dr. C. Laane.

4.4 RESULTS

Occurence of different flavodoxins in A.vinelandii

Flavodoxin isolated from N_2-fixing A.vinelandii cells appears to be heterogeneous by two dimensional gel electrophoresis (Fig. 1A). The purified flavodoxin was separated into two protein spots (indicated I and II). Spot I has a pI of 4.90 and a M_r of 20500. Spot II has a pI of 4.94 and a M_r of 19500. Flavodoxin isolated from cells grown on NH_4Ac also separates into different spots on two dimensional gels (Fig. 1B). Spot I and spot II are identical to spot I and spot II in flavodoxin from N_2-fixing cells as was shown on a two dimensional gel of a mixture of the flavodoxins from N_2-fixing cells and NH_4^+-grown cells (not shown). Spot III has a pI of 4.60 and a M_r of 20500.

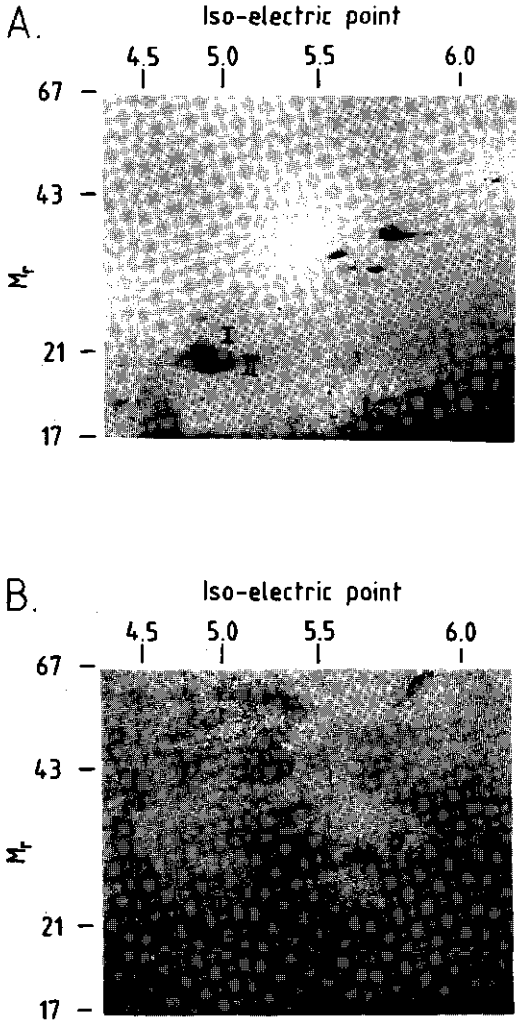

Fig. 1. Two dimensional gels of isolated flavodoxins.
Flavodoxin was isolated as described in Materials and Methods and analyzed by two dimensional gel electrophoresis [31]. The pH gradient is indicated at the top, the position of molecular mass markers (in kDa) at the left of each gel. Spots derived from flavodoxin (marked I, II, III) were verified later using FPLC purified flavodoxin fractions (A) 10 µg flavodoxin isolated from cells grown N_2-fixing. (B) 10 µg flavodoxin isolated from cells grown on NH_4Ac.

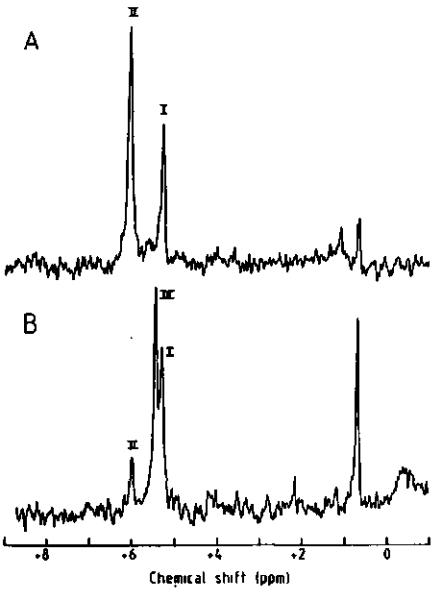

Fig. 2. ^{31}P-NMR spectra of flavodoxins isolated from N_2-fixing and from NH_4Ac grown cells.
121 MHz ^{31}P-NMR spectra of Azotobacter flavodoxins were recorded in 50 mM Tris-HCl, 1 mM EDTA, final pH 7.5. A 3.0 Hz exponential multiplication was applied before Fourier transformation. (A) 1 mM flavodoxin from cells grown N_2-fixing; 3662 acquisitions. (B) 0.2 mM flavodoxin from cells grown on NH_4Ac; 35300 acquisitions.

The heterogeneity of the flavodoxin samples was confirmed by ^{31}P-NMR spectroscopy (Fig. 2). The ^{31}P-NMR spectrum of purified flavodoxin from N_2-fixing cells indicated that two different flavodoxins were present. One with a chemical shift for the phosphate of bound FMN of 5.33 ppm. One with a chemical shift of 6.05 ppm. The ^{31}P-NMR spectrum of purified flavodoxin from cells grown on NH_4Ac revealed the presence of three different flavodoxins, with chemical shifts for the phosphate of the FMN moiety of 5.35, 5.48, 6.03 ppm. One of the three flavodoxins also contains a phosphor residue (δ = 0.8 ppm) linked to the protein moiety. Signal intensities indicate that the phosphor residue is most likely attached to flavodoxin I and/or III. In contrast to earlier reports [10,11] no significant amount of protein bound phosphate (apart from the phosphate group of FMN) was detectable in flavodoxin from N_2-fixing cells.

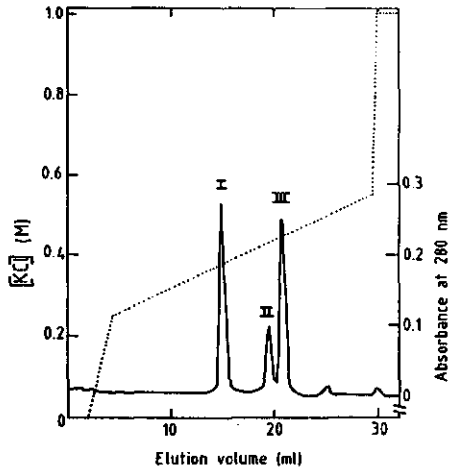

Fig. 3. Separation of flavodoxin I, II and III on Mono Q.
0.5 mg flavodoxin isolated from cells grown on NH_4Ac was separated on Mono Q HR5/5 with a linear salt gradient by FPLC as described in Materials and Methods. Flow rate: 1.5 ml/min. The solid line represents the A280 and the dashed line the KCl concentration.

The three flavodoxins present in the mixture isolated from cells grown on NH_4Ac can be separated by using FPLC (Fig. 3). Flavodoxin I elutes at 0.38 M KCl, flavodoxin II at 0.43 M KCl, flavodoxin III at 0.45 M KCl. Flavodoxin of N_2-fixing cells separates in the same way, but yields considerably more Fld II and no Fld III. The separated flavodoxins were pure as judged by two dimensional gel electrophoresis and ^{31}P-NMR spectroscopy. In fact the nomenclature used in Figs. 1 and 2 is based on the elution profile of the different flavodoxins from the FPLC. However the protein-bound phosphate group with a chemical shift of 0.8 ppm was not found in any of the flavodoxin fractions after FPLC, for unknown reasons.

Characterization of the different flavodoxins

It turned out during this investigation that the properties of Fld I and Fld II were the same irrespective whether the fractions were isolated from cells grown on NH_4Ac or from cells grown N_2-fixing. Therefore

in the rest of this paper the origin of flavodoxins I and II is not specified.

The iso-electric points of Fld I, II and III in 9 M urea were determined by two dimensional gel electrophoresis [31]. Their relative

Table 1. Properties of Azotobacter flavodoxins.

	Flavodoxin I	Flavodoxin II	Flavodoxin III
pI in urea	4.95 ± 0.05	5.05 ± 0.1	4.55 ± 0.1
Mr (SDS-gels)	21500 ± 1000	20500 ± 1000	21500 ± 1000
^{31}P chemical shift FMN	5.34	6.05	5.50
λ_{max} FMN (quinone) (nm)	458	452	461
λ_{max} FMN (semiquinone) (nm)	599	581	592
Absorbance ratio*	5.3 (274/458)	4.9 (274/452)	4.3 (274/461)
$\epsilon^*(mM^{-1}cm^{-1})$ quinone	11.6 (458)	11.3 (452)	10.6 (461)
semiquinone	3.0 (458)	3.0 (452)	3.1 (461)
	4.8 (599)	5.7 (581)	5.4 (592)
hydroquinone	1.2 (458)	1.5 (452)	1.5 (461)
E1 (pH 7.0; 25°C) (mV)	-320 ± 10	-500 ± 10	-500 ± 10
Concentration in			
N_2-fixing cells (μM)	50 ± 20	100 ± 20	0
NH_4^+ grown cells (μM)	36 ± 15	10 ± 5	30 ± 10
Oxidized by nitrogenase?	no	yes	not tested
formed during nif derepression?	no	yes	no

* Wavelengths (nm) in parenthesis.

molecular masses by SDS-polyacrylamide electrophoresis using marker proteins as described in Materials and Methods. Mean values of 5 independent determinations are given in Table 1.

Spectra of the quinone forms of flavodoxins I, II and III are shown in Fig. 4. Fld I has absorption maxima at 274, 378, 458, 479 nm; Fld II at 274, 372, 452, 471 nm and Fld III at 274, 371, 461, 481 nm. Absorp-

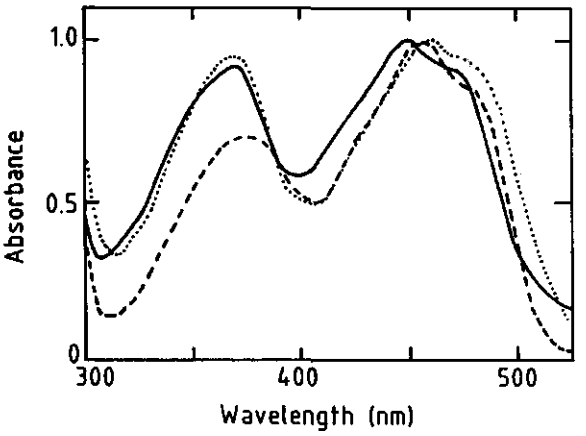

Fig. 4. Spectra of the quinone forms of flavodoxins I, II and III. Spectra were recorded in 50 mM Tris-Cl, 0.1 mM EDTA, final pH 7.5 and normalized to the absorption maxima at 450 nm. Fld I is shown as a broken line. Fld II as a solid line. Fld III as a dotted line.

tion maxima for the semiquinone form (not shown) of Fld I are 599, 635 nm; Fld II 581, 615 nm and Fld III 592, 625 nm. During reduction of the quinone to the semiquinone form an isobestic pont occurs at 504 nm for Fld I; at 500 nm for Fld II and at 514 nm for Fld III.

The extinction coefficients determined at the absorption maxima of the FMN (λ_{max}) around 450 nm are almost similar for the three proteins (Table 1). One exception is the ϵ_{599} for the semiquinone form of Fld I, which is low compared to Fld II and Fld III. Until now the concentration of the quinone form of Azotobacter flavodoxin was always determined by its absorbance at 452 nm and for the semiquinone form at 580 nm. The ratio A274/A452 was used as a measure of the purity of flavodoxin. As can be seen in Table 1 it is better to do this at different wavelengths, depending on the type of flavodoxin.

The data of Table I were used in the determination of the redox potentials of the three flavodoxins. For Fld II and III the redox pontential of the semiquinone/hydroquinone couple (E1) was determined with hydrogen and hydrogenase. No striking differences were found between the redox potentials of Fld II and Fld III. The effect of pH on E1 is shown in Fig. 5. The change in slope at pH values above 7 indicates a deproto-

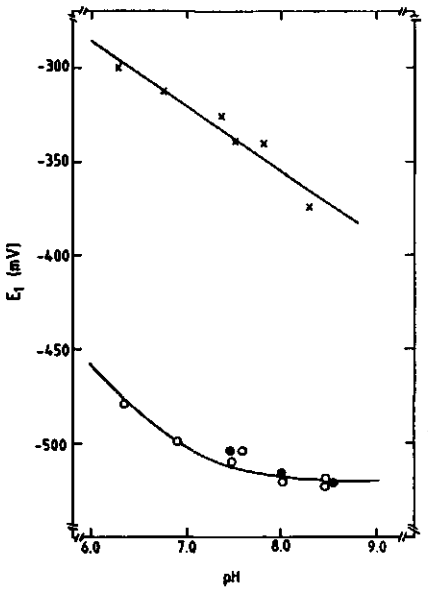

Fig. 5. Effect of pH on E1 of flavodoxins I, II and III.
The redox titrations were performed at 25°C as described in Materials and Methods. (x); measurements for Fld I. (●); for Fld II. (o); for Fld III.

nation in flavodoxin hydroquinone. Through the experimental data a theoretical curve is drawn in which the oxidation/reduction potential of the unprotonated flavodoxin is -520 mV and the pK of the reduced form is 7.0 [28]. The midpoint potential at pH 7.0 calculated from this theoretical curve is -500 mV for both Fld II and Fld III. Between pH 6.0 and 8.0 Fld I was always completely in the hydroquinone form when measured with hydrogen and hydrogenase. Therefore E1 of Fld I was measured with NADPH/$NADP^+$ and ferredoxin-$NADP^+$ oxidoreductase (Fig. 5). At pH 7.0 E1 of Fld I is -320 mV, a value much more positive than E1 of Fld II or Fld III. The pH dependence of E1 for Fld I is abnormal for a flavodoxin. The curve drawn through the experimental data equals an n = 1.7. Maybe this unusual behaviour is caused by Fld I having more than one ionization in the measured pH range that influence E1. Another possibility is that both redox couples of Fld I are not well separated.

Relationship of flavodoxins

The three flavodoxins of A.vinelandii can either be structurally different polypeptides or be modified forms of the same polypeptide chain. The most straightforward, but also most difficult, way to study the relationship of flavodoxins I - III would be comparison of their amino acid sequences. A more easy method for peptide analysis of proteins is peptide mapping [32]. Fld I, II and III were partially digested with either protease from Staphylococcus aereus V8 or with chymotrypsin. Products were analyzed on 20% SDS-polyacrylamide gels (Fig. 6). The pat-

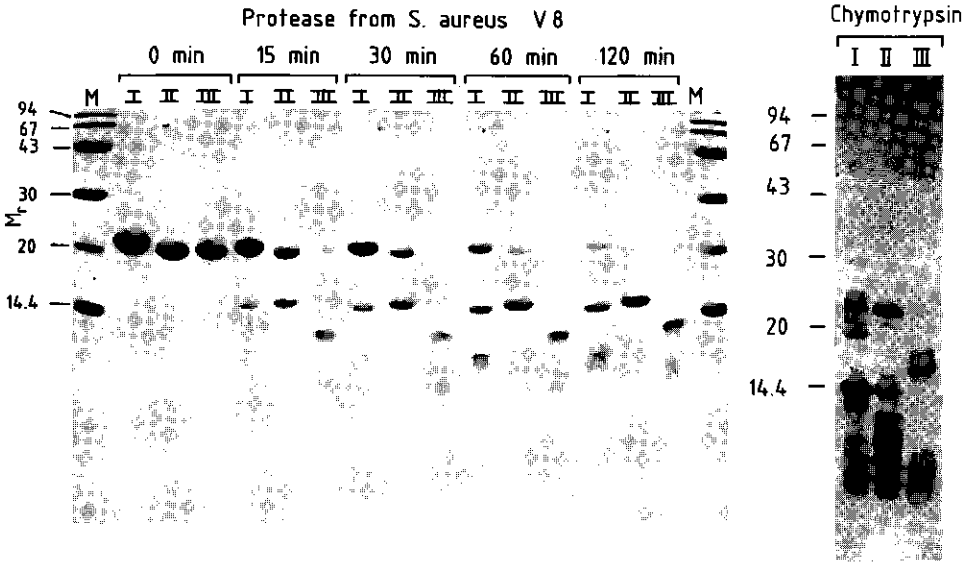

Fig. 6. Peptide mapping of Fld I, II and III.
Flavodoxins I, II and III (1 mg/ml) were denatured in 0.4% SDS and digested at room temperature with 40 µg /ml protease from S.aureus V8 for 0-120 min as indicated in the figure or with 15 µg/ml chymotrypsin for 60 min. 10 µg protein was analyzed on a 20% gel according to Laemmli [29] or in the case of chymotrypsin with 0.07% bisacrylamide instead of 0.53%. The position of molecular mass markers (M) after electrophoresis is indicated in kDa.

tern of peptide fragments produced were sufficiently different to conclude that Fld I, II and III are encoded by different genes. To what extent there is difference between the primary structures of the three proteins cannot be concluded from the peptide maps shown in Fig. 6.

Another way to obtain information about the relationship of proteins is comparison of their immunological properties. Antisera were raised separately against the three flavodoxins. The reaction of these antisera with the three flavodoxins was compared (Table 2). The cross-

Table 2. Cross reactivity of anti-Fld I, II and III with Fld I, II and III.

The reactivity of the antisera was measured as described in Materials and Methods and is expressed as ^{125}I-counts per minute bound to the purified proteins.

Sample	Amount of Protein	Reactivity with antisera against		
		Fld I	Fld II	Fld III
	µg	^{125}I-counts.min^{-1}		
Control	0	72	59	63
Flavodoxin I	0.2	7326	146	61
Flavodoxin II	0.25	379	14998	107
Flavodoxin III	0.2	65	61	1390

reactivity of an antiserum raised against one type of flavodoxin with another type of flavodoxin was always less than 5%. Such small cross-reactivity could easily be caused by the presence of trace amounts of the other flavodoxins in the "pure" flavodoxin preparations used to raise antibodies. Anyhow Table 2 strongly suggests that flavodoxins I, II and III are completely different immunological structures.

Flavodoxins I, II and III in whole cells

The loss of covalently bound phosphate for one of the "ammonia" flavodoxins after FPLC raised questions about the physiological relevance of the purified flavodoxins. The antisera raised against the three flavodoxins were used to investigate whether Fld I, II, III were present in a modified form in the intact cells. Proteins from fermentor grown cells were separated by two dimensional gel electrophoresis. Protein blots were made form these gels and the positions of Fld I, II and III detected with their own specific antisera [21]. The position of the flavodoxins in the extracts could then be compared with the position of pure flavodoxins on two dimensional gels. The positions of Fld I and Fld II after electrophoresis turned out to be exactly the same compared to the positions of purified Fld I and Fld II (not shown). So the purified Fld I and Fld II seem to be identical to the native proteins. On two dimensional gels the apparent Mr of purified Fld III was higher than the Mr observed for Fld III present in cell extracts (Fig. 7). Obviously during purification the structure of this protein is altered.

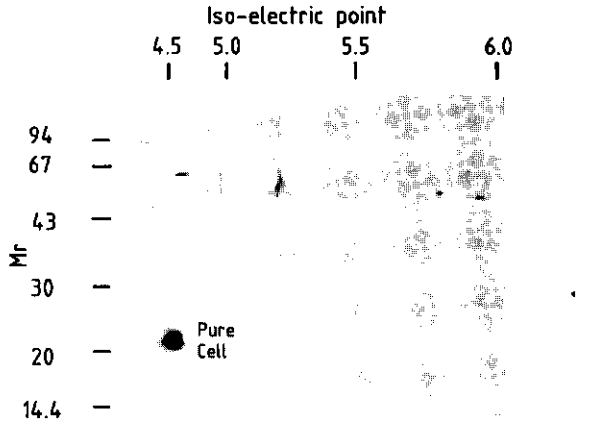

Fig. 7. Immunoautoradiogram of purified Fld III and Fld III present in intact A.vinelandii cells.
Two dimensional gel electrophoresis was done with a mixture of purified Fld III (0.05 µg) and an extract from cells (18 µg protein) grown on NH_4Ac. The pH gradient is indicated at the top, the position of molecular mass markers (in kDa) at the left side of the gel. After electrophoresis a protein blot was made and the blot incubated with antiserum against Fld III [21]. Bound antibodies were detected with ^{125}I-protein A and autoradiography. The positions of purified Fld III (pure) and Fld III as it occurs in the cells (cell) are indicated.

In a previous paper [21] we estimated the concentration of flavodoxin in intact A.vinelandii cells. At that time no discrimination was made between different types of flavodoxins. Furthermore the antiserum used, reacted predominantly with flavodoxin II. Therefore the concentration of flavodoxin in intact cells was reinvestigated using specific antisera against flavodoxins I, II and III. Instead of growing cells in a chemostat, cells were grown logarithmically in a fermentor. The concentration of flavodoxin was calculated as described earlier [21] using a M_r for flavodoxin of 20000 and an internal volume for Azotobacter cells of 5 μl/mg total protein (Table 1). The concentration of flavodoxin I is almost equal for cells grown N_2-fixing compared to growth on NH_4Ac. Whereas the concentration of Fld II is ten times higher in N_2-fixing cells. Fld III is only detectable during growth on NH_4Ac.

As mentioned earlier [21] synthesis of flavodoxin parallels synthesis of the nitrogenase proteins after derepression (transfer to N_2 dependent growth). So at least part of the flavodoxin present in N_2-fixing A.vinelandii cells seem to be nif-specific. This is most likely flavodoxin II. To prove this, cells were derepressed in the presence of $^{35}SO_4^{2-}$. Flavodoxin present in extracts from these cells was precipitated with anti-Fld I and anti-Fld II, followed by SDS-gel electrophoresis and autoradiography. The autoradiogram showed, that during derepression only Fld II was synthesized. No ^{35}S-labelled Fld I was detectable (not shown). In a similar experiment, using an antiserum against ferredoxin I, it appeared that no significant amount of ferredoxin I was synthesized during derepression (not shown).

4.5 DISCUSSION

In the introduction of this paper the properties of Azotobacter flavodoxin are described as if it concerns only one protein. In this report it has been shown, that A.vinelandii is able to synthesize at least three different flavodoxins. All three flavodoxins are present in cells grown on NH_4Ac. In cells grown N_2-fixing only Fld I and Fld II are

found. The derepression experiments followed by immunoprecipitation and also the high concentration in N_2-fixing cells, indicate that Fld II is involved in nitrogen fixation. Since Fld II, when properly reduced, can donate efficiently electrons to nitrogenase (J. Cordewener, unpublished), it is most likely that Fld II can act as a physiological electron donor for nitrogenase. Fld II is also present to a small extent in NH_4^+-grown cells (10%). This might be caused by the presence of a constitutive promotor for the same protein in addition to a nif promotor. Such a phenomenon has been described for glutamine synthetase in Anabaena 7120 [36].

One could wonder why the heterogeneity of flavodoxin has not been reported earlier. Firstly, it is important to notice that flavodoxins I, II and III are difficult to separate by conventional methods. For this reason the mixture is easily regarded as one single protein. For instance Dickerson et al. [37] used a preparation that gave two bands on a SDS-polyacrylamide gel. It might be possible that these two bands represent Fld I and Fld II, but the authors have interpreted the splitting of the flavodoxin band as being due to the formation of an internal cross-link [37]. Secondly, it is possible that there are differences between A.vinelandii strains with respect to the production of the three different flavodoxins. Maybe some strains only produce one type of flavodoxin under N_2-fixing conditions. While from A.vinelandii ATCC 478 always a mixture of Fld I and II has been isolated, it is possible to purify flavodoxin II from A.vinelandii OP by conventional methods (W.J.H. van Berkel, unpublished). This flavodoxin gives one FMN peak in the ^{31}P-NMR spectrum. The ^{31}P-NMR spectrum published for flavodoxin isolated from A.vinelandii OP (Berkeley) also shows that the preparation used contains one type of flavodoxin [10,11]. ^{31}P-NMR spectroscopy reveals another difference. Apart from the FMN, Fld II isolated from A.vinelandii ATCC 478 had no protein bound phosphate, but in flavodoxin isolated from A.vinelandii OP one bound phosphate was detected. (J. Vervoort, personal communication). For A.vinelandii OP (Berkeley) two phosphate groups, bound to Fld II in addition to the FMN phosphate were observed [10,11]. That Azotobacter flavodoxin might be different depending on the strain used to isolate the protein was already suggested by Yoch [9] in 1975. He isolated a flavodoxin from

Azotobacter that differed from the known Azotobacter flavodoxins and proposed to call his particular strain A.vinelandii strain OP (Berkeley). The difference between the primary structure of this strange flavodoxin [8] and for instance the "normal" flavodoxin from A.vinelandii strain O [38] however is very small, being a substitution of glutamate for aspartate at positions 104, 134, 136, 139.

Most results until now have been obtained with flavodoxin from A.vinelandii OP (Berkeley) [4,8-13,17]; strain OP [1-3,14,20]; strain O [5,15,38]. We think that flavodoxin II is similar in all A.vinelandii strains used, but that differences arise depending upon the degree of phosphorylation of flavodoxin II and upon the contamination of purified Fld II with Fld I. For instance it is possible that the anomalous redox potential for E2 [15] of +50 mV was measured with a mixture of two flavodoxins and one flavodoxin had anomalous redox properties. In this respect it is interesting to note a recent report on the isolation of flavodoxin from A.vinelandii mutant TZN 200 [39]. This flavodoxin had a higher redox potential (E_1) than the wild-type flavodoxin and a modified structure of the FMN. Although the properties are somewhat different, the mutant flavodoxin might well be similar to our Fld I. In that case a different explanation for the results of Hofstetter and DerVartanian [39] is that synthesis of Fld II is repressed in the mutant strain A.vinelandii TZN 200 leading to the isolation of pure Fld I, whereas isolation from the wild-type gives predominantly Fld II.

Earlier experiments reported from our laboratory with flavodoxin from A.vinelandii ATCC 478 [16,19,21,40-42] were performed with a mixture of 35% Fld I and 65% Fld II. Since Fld I is not oxidized by nitrogenase (E_1 is too positive), the published value of -495 mV for the midpoint of the redox potential at which flavodoxin transfers electrons to nitrogenase [19,40] is too negative. When the redox dependency of flavodoxin oxidation by nitrogenase is measured with pure flavodoxin II, this potential shifts to a more positive value of -475 mV (J. Cordewener, unpublished). Another experiment that can be explained differently now is the reduction of flavodoxin to the hydroquinone form by NADPH with purified NAD(P)H-flavodoxin oxidoreductase [42]. The hydroquinone that is formed, originates most likely from Fld I (E1 = -320 mV), while Fld II is not reduced beyond the semiquinone state (E_1 = -500

mV, E2 = -250 mV). So the presence of an enzyme system capable of reducing flavodoxin II to the hydroquinone form and consequently electron transport to nitrogenase, is again an open question. Because both Fld II and Fld III have a low redoxpotential, it may well be that the enzymes necessary to reduce Fld II or III are identical for N_2-fixing and NH_4^+-grown cells. In that case part of the electron transport to nitrogenase viz. the flavodoxin II reducing system is not nif specific.

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5. STUDIES ON THE MECHANISM OF ELECTRON TRANSPORT TO NITROGENASE IN *AZOTOBACTER VINELANDII*

5.1 SUMMARY

The involvement of the cytoplasmic membrane in electron transport to nitrogenase has been studied. Evidence is shown that nitrogenase activity in Azotobacter vinelandii is coupled to the flux of electrons through the respiratory chain.

To obtain information about proteins involved, the changes occurring in A.vinelandii cells transferred to nitrogen-free medium after growth on NH_4Cl (derepression of nitrogenase activity) were studied. Synthesis of the nitrogenase polypeptides was detectable 5 min after transfer to nitrogen-free medium. No nitrogenase activity could be detected until t=20 min, whereupon a linear increase of nitrogenase activity with time was observed. Synthesis of nitrogenase was accompanied by synthesis of flavodoxin II and two membrane bound polypeptides of M_r 29000 and 30000. Analysis with respect to changes in membrane-bound NAD(P)H dehydrogenase activities revealed the induction of an NADPH dehydrogenase activity, which was not detectable in membranes isolated from cells grown in the presence of NH_4Ac. This induced activity was associated with the appearance of a polypeptide of M_r 29000 in the NADPH dehydrogenase complex.

5.2 INTRODUCTION

The enzyme nitrogenase is capable of reducing atmospheric N_2 to ammonia. For activity the enzyme needs an anaerobic environment, MgATP and a strong reductant. How this reductant is generated in the aerobic nitrogen-fixing bacterium Azotobacter vinelandii is not well understood. In 1971 Benemann et al. [1] proposed a linear electron transfer pathway from NADPH to nitrogenase, including both ferredoxin and flavodoxin. However the nitrogenase activity measured with endogenous proteins was less than 5% of the activity with dithionite as electron donor. Haaker et al. [2,3] criti-

sized, that the proposed model is too simplistic with respect to the redox potentials of the different components of the electron transfer chain. It has been shown by the same authors [4] that a high proton motive force across the cytoplasmic membrane is required for nitrogenase activity. Especially the membrane potential turned out to be an important factor [5]. Recently it has been shown that A.vinelandii is able to make at least three different flavodoxins [6]. Flavodoxin II is most likely the reductant for nitrogenase [6]. Whether ferredoxin is involved in electron transport to nitrogenase in vivo is uncertain [6,7]. An enzyme system capable of reducing flavodoxin II has still to be found.

This paper describes the changes observed in A.vinelandii cells transferred to nitrogen-free medium after growth in the presence of NH_4Cl. For Azotobacter species it is known that, when sufficient NH_4^+ is supplied to cells, synthesis of the nitrogenase proteins [8] and possibly also 13 other nif specific polypeptides [9] is repressed. It has been investigated whether during derepression, the (membrane) proteins necessary for electron transport to nitrogenase are synthesized simultaneously with the nitrogenase proteins. In addition, a comparison has been made between the membrane bound NAD(P)H dehydrogenases from cells grown in the presence of NH_4Ac and N_2-fixing cells.

The involvement of the cytoplasmic membrane in nitrogen fixation has been studied on the analogy of work of Elferink et al. [10,11]. These authors proved, that active solute transport in certain bacteria was not only dependent on the membrane potential, but also on the flux of electrons through a cyclic or a linear electron transport chain. In the present report a relationship between nitrogenase activity of A.vinelandii and the rate of electron transport through the respiratory chain will be demonstrated.

5.3 MATERIALS AND METHODS

Derepression of nitrogenase activity

Azotobacter vinelandii ATCC 478 was grown in a chemostat at a dilution rate of 0.15 h^{-1}, with sucrose as carbon source and 28 mM NH_4Cl as N-source [12]. Cells (100 ml) were harvested and washed two times with 250 ml

nitrogen-free medium. Cells (10 ml) were incubated at the original density (0.7 mg protein.ml^{-1}) at 30°C in Burk's nitrogen-free medium in the system described by Haaker et al. [4]. Nitrogenase activity was measured as acetylene reduction. Newly synthesized proteins were labelled by incubating cells for a certain time interval, in a medium containing 25 µCi [^{35}S]methionine or 100 µCi $Na_2{}^{35}SO_4$ (New England Nuclear). After the addition of 1 mg/ml methionine or 50 mM Na_2SO_4, the cells were centrifugated, washed with 10 ml 10 mM Tris-Cl, 10 mM EDTA, final pH 8.0 and boiled in either 1 ml sample buffer for electrophoresis or suspended in 10 ml 50 mM Tes-NaOH, 5 mM $MgSO_4$, final pH 7.5 for the isolation of membranes. As a control, the same experiments were performed with 28 mM NH_4Ac present in the incubation mixture.

Isolation of membranes

Cells, suspended in 50 mM Tes-NaOH, 5 mM $MgSO_4$, final pH 7.5 were broken at 0°C by sonication for four 30-s periods in a MSE sonifier. The membranes were sedimented by centrifugation between 30 min 20000 x g and 60 min 200000 x g. The pellet was suspended in 50 mM Tes-NaOH, 5 mM $MgSO_4$, final pH 7.5 to the original volume, sonicated for two 10-s periods and centrifuged as before. Washed membranes were either boiled in sample buffer for electrophoresis at a protein concentration of 5 mg/ml or suspended in 50 mM Tes-NaOH, 5 mM $MgSO_4$, final pH 7.5 at a protein concentration of 40 mg/ml and stored at -80°C.

Analytical methods

Protein concentrations were estimated with the Lowry method [13]. SDS-polyacrylamide gel electrophoresis was carried out according to Laemmli [14]. Gels contained 14% or 17.5% acrylamide with 0.09% or 0.07% bisacrylamide respectively. For molecular mass markers see [6]. Nitrogenase activity of whole cells was measured at different oxygen input rates as described earlier [15]. The maximum activity is given in the text. Nitrogenase activity in vitro was measured with dithionite and an ATP regenerating system after making cells permeable for small molecules with hexadecyltri-

methylammonium bromide [16]. Maximum activities were obtained at 0.8 mg/ml protein and 0.14 mg/ml hexadecyltrimethylammonium bromide.

The membrane potential was measured with an ion-selective electrode as described [10,11].

Rates of respiration were measured at 30°C in a Gilson oxygraph equiped with a Clark-type O_2 electrode at 15 µg/ml protein in 50 mM Tes-NaOH, 10 mM $MgAc_2$, 20 µg/ml catalase, final pH 7.4.

Nitrobluetetrazolium (Sigma Chemical Co.) reduction was measured spectrophotometrically at 560 nm. Activities were measured at 30°C at 20 µg protein/ml in 50 mM Tes-NaOH, 5 mM $MgSO_4$, 20 µg/ml catalase, 0.5 mM nitrobluetetrazolium, final pH 7.5.

For non-denaturing gel electrophoresis, membranes (8 mg protein/ml) were solubilized in 8% triton X-100, 50 mM Tris-Cl pH 8.8, 2 mM $MgCl_2$, 10% glycerol. Solubilized proteins (50 µg/well) were separated on 7.5% polyacrylamide gels containing 0.17% bisacrylamide [14], in which SDS was replaced by triton X-100. After electrophoresis, gels were stained with 0.2 mM NAD(P)H, 0.5 mM nitrobluetetrazolium in 50 mM Tes-NaOH, 5 mM $MgSO_4$, final pH 7.5.

5.4 RESULTS

Nitrogen fixation and respiration

It is known that the energized cytoplasmic membrane is involved in the generation of reducing power for nitrogen fixation. The proton motive force in A.vinelandii is generated by respiration and it has been shown that whole cell nitrogenase activity is dependent on a high membrane potential [5]. To obtain more information about the influence of the membrane potential on whole cell nitrogenase activity, both were measured simultaneously at different oxygen input rates. At an oxygen input rate of zero no nitrogenase activity was detectable. When the oxygen input rate was increased both the membrane potential and the nitrogenase activity increased to a maximum. When the oxygen input rate was further increased the membrane potential remained at the maximum, but the nitrogenase activity declined.

To measure the membrane potential of A.vinelandii cells the outer cell wall has to be removed by a Tris-EDTA treatment [11]. It was striking that this treatment caused a 90% loss of both the nitrogenase activity and the respiration rate of cells. For this reason no absolute data on the magnitude of the membrane potential are presented here. The observed simultaneous loss of nitrogenase activity and the rate of respiration led us to study a possible relationship between the two processes. The rate of respiration of A.vinelandii cells depends on the oxygen input during growth and also on the carbon source used during the experiment (Table 1). The

Table 1. Respiration rates and nitrogenase activities of A.vinelandii cells.

A.vinelandii cells were cultured with sucrose as carbon source in a chemostat at a dilution rate of 0.19 h^{-1} and harvested. Nitrogenase activities and respiration rates of whole cells were measured with different carbon sources as described in Materials and Methods.

Substrate	Respiration rate (μmoles O_2 consumed min^{-1}.mg $protein^{-1}$)	Nitrogenase activity (nmoles C_2H_4 formed. min^{-1}.mg $protein^{-1}$)
Pyruvate(20 mM)	0.40	52
Glucose (60 mM)	0.63	69
Acetate (20 mM)+ glucose(20 mM)	1.17	127
Sucrose (60 mM)	1.16	127
Fructose(60 mM)	1.63	155

respiration rate of sucrose grown cells was low with pyruvate and high with fructose; a similar relationship was found with the nitrogenase activity. As can be deduced from Table 1 the nitrogenase activity seems to be related with the respiration rate. A similar relationship was found when the nitrogenase activity and the rate of respiration were followed during growth of cells in batch culture (Fig. 1). At an early stage of growth, the respiration rate of cells was high as was the nitrogenase activity. At higher densities, growth became oxygen limited and the respiration rate of the cells decreased. The nitrogenase activity decreased proportionally (Fig. 1).

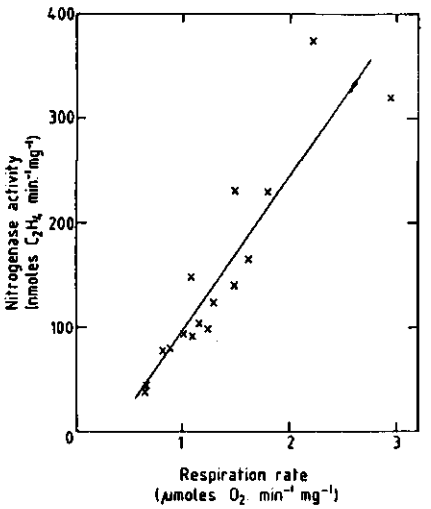

Fig. 1. Relationship between the respiration rate of cells and the nitrogenase activity.
Cells were grown in batch cultures on nitrogen-free medium. During growth, samples were taken to measure the respiration rate of the cells and whole cell nitrogenase activity as described in Materials and Methods. The line drawn through the data points is a least squares fit.

Derepression of nitrogenase activity

To obtain information about the proteins involved in electron transport derepression experiments were performed. When A.vinelandii cells, grown on NH_4Cl, are transferred to nitrogen-free medium, the enzymes necessary for nitrogenase activity are synthesized during the first hour(s) of incubation. Nitrogenase activity (C_2H_2 reduction) is detectable after 20 min at earliest, whereupon a linear increase in activity with the incubation time is measured (Fig. 2). The observed increase in activity may reflect the synthesis of active nitrogenase enzyme; however it is also possible that active nitrogenase is already present in the cells at an earlier stage of the incubation, and that this relationship reflects the synthesis of the electron transport pathway to nitrogenase. To discriminate between both

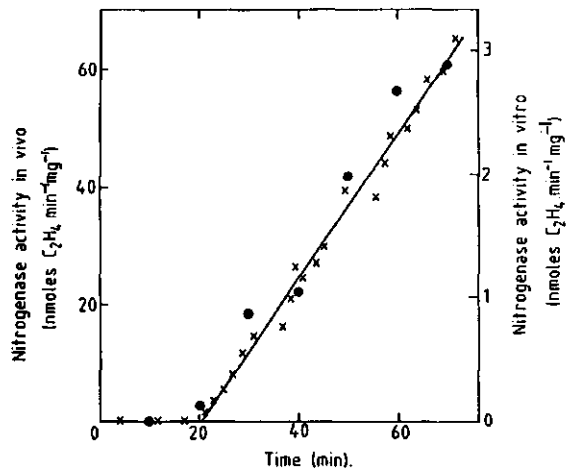

Fig. 2. Derepression of nitrogenase activity in A.vinelandii. A.vinelandii cells were cultured in a chemostat with 28 mM NH_4Cl in the medium. After washing, cells were transferred to nitrogen-free medium as described in Materials and Methods. Cells were incubated at a free oxygen concentration of 2 μM. During the incubation, nitrogenase activity (C_2H_2 reduction) was measured with intact cells (x, in vivo) and with cells made permeable for dithionite and ATP (•, in vitro).

possibilities, the nitrogenase activity in vivo has been compared to the nitrogenase activity measured in vitro with dithionite and an ATP regenerating system as described earlier [16] (Fig. 2). By using this method nitrogenase activity in vitro can be measured immediately after taking a sample out of the incubation mixture. Although activities are low, the experiment shown in Fig. 2 indicates, that the nitrogenase activity in vivo and in vitro increase synchronously. Consequently, during derepression, electron transport to nitrogenase is either not rate-limiting for the activity in vivo or it is made exactly at the same rate as active nitrogenase is synthesized.

Protein synthesis during derepression is shown in Fig. 3. Synthesis of the Fe-protein of nitrogenase (Av_2) is observed already during the first 5 min after transfer of cells from a NH_4Cl containing medium to nitrogen-free medium. The first detectable synthesis of the two polypeptides of Mo-Fe protein (Av_1) occurs between 5-10 min. As reported earlier [6] the only

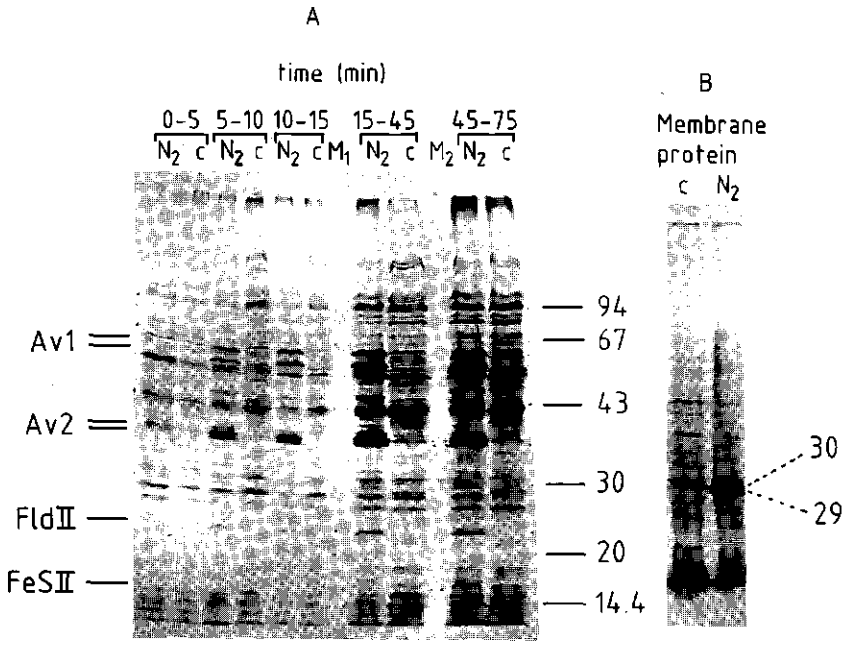

Fig. 3. Protein synthesis during derepression.
A.vinelandii cells were cultured in a chemostat with 28 mM NH_4Cl in the medium. After washing, cells were transferred to nitrogen-free medium (N_2) or medium containing 28 mM NH_4Ac (c). At different times samples were assayed for protein synthesis by addition of [^{35}S]methionine to the incubation. Labelled proteins (100 µg/well) were analyzed on 14% gels as described in Materials and Methods. The positions of molecular mass markers after electrophoresis are indicated in kDa. (M1) Mixture of molecular mass markers, Fld II and Fe/S II; (M2) mixture of the nitrogenase proteins Av_1 and Av_2, Fld II and Fe/S II. (A) Whole cells, ^{35}S-labelled as indicated. (B) Membrane proteins, ^{35}S-labelled between 0-50 min. The dashed lines indicate the positions of the 29 kDa and 30 kDa proteins.

derepressible protein synthesized in significant amounts simultaneously with the nitrogenase proteins is flavodoxin II, one of the three different flavodoxins present in A.vinelandii (Fig. 3A). The rates of synthesis of all other polypeptides, particularly that of Fe/S protein II and also those possible involved in electron transport, are equal to the rate of synthesis in a control experiment (Fig. 3A). However analysis of membrane proteins revealed two derepressible polypeptides of M_r 29000 and 30000 (Fig. 3B). Because membranes constitute only a small portion of the whole cell, these protein bands are hardly visible in Fig. 3A.

Membrane bound NAD(P)H dehydrogenases

Because of the observed relationship between whole cell nitrogenase activity and the rate of respiration and because of the induction of membrane bound proteins during derepression, the cytoplasmic membrane bound pyridine nucleotide dehydrogenases have been studied. The properties of NAD(P)H dehydrogenases in membranes from N_2-fixing cells were compared with those in membranes isolated from cells grown in the presence of NH_4Ac. Since transhydrogenase is a soluble enzyme in A.vinelandii [17] and thoroughly washed membranes have been used, the activities cannot be due to transhydrogenase activity. No differences are observed with respect to electron transfer rate from NAD(P)H to oxygen between both types of membranes (Table 2). The activities are comparable to published values for membranes from N_2-fixing cells [18,19]. In addition to measurement of the

Table 2. NAD(P)H dehydrogenase activities of Azotobacter membranes.

Membranes were isolated and the oxygen uptake rates as well as the tetrazolium reduction rates were measured with different substrates as described in Materials and Methods. NH_4^+ = membranes isolated from cells grown in the presence of 28 mM NH_4Ac. N_2 = membranes isolated form N_2-fixing cells.

Substrate	Addition	Oxygen consumption		Tetrazolium reduction rates	
		NH_4^+	N_2	NH_4^+	N_2
		μmoles O_2 consumed. min^{-1}.mg $protein^{-1}$		μmoles tetrazolium reduced.min^{-1}.mg $protein^{-1}$	
1 mM NADH	none	2.40	2.75	0.63	0.53
1 mM NADH	1 mM NAD^+	1.80	1.90	0.60	0.55
2 mM NADPH	none	2.30	2.20	0.25	0.20
2 mM NADPH	1 mM NAD^+	0.40	0.40	0.24	0.22

rate of electron transport to oxygen through the respiratory chain, NAD(P)H tetrazolium reductase rates were measured [20]. The differences between membranes isolated from N_2-fixing cells and membranes from cells grown in

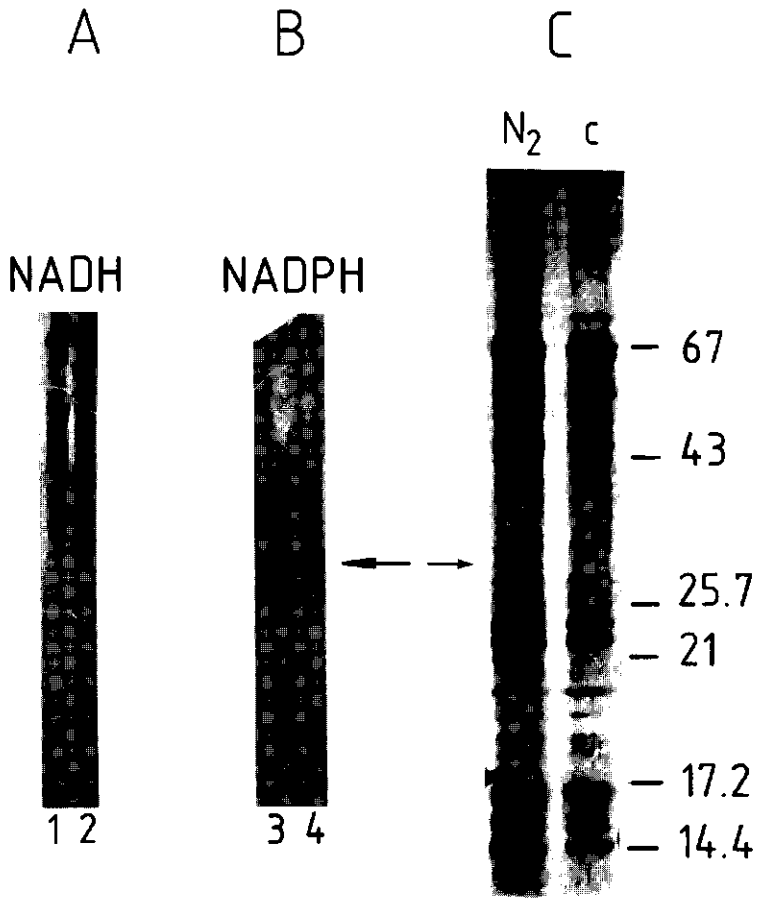

Fig. 4. Gel electrophoresis of membrane bound NAD(P)H dehydrogenases. Membrane proteins were solubilized in triton X-100 and separated on polyacrylamide gels in the absence of SDS. After electrophoresis the gels were either stained for NADH (A) or NADPH (B) tetrazolium reductase activity as described in Materials and Methods. (1,3) Membranes isolated from cells grown in the presence of NH_4Ac; (2,4) membranes isolated from cells grown N_2-fixing. The arrow in part B indicates a NADPH tetrazolium reductase predominantly observed in membranes after derepression of nitrogenase activity. Proteins present in this band were extracted, separated on a 17.5% SDS-polyacrylamide gel and silver stained [21] (C). The positions of molecular mass markers after electrophoresis are indicated in kDa. (N_2) polypeptides extracted from lane 4; (c) control i.e. polypeptides extracted from lane 3 at the position of the arrow in part B. The arrrow in part C points to a 29 kDa polypeptide solely observed in the dehydrogenase complex originating from N_2-fixing cells.

the presence of NH_4^+ are marginal (Table 2). Note that although NAD^+ is a strong inhibitor of electron transfer from NADPH to O_2, the rate of tetrazolium reduction by NADPH is not inhibited by NAD^+ (Table 2).

When membranes are incubated with triton X-100, 90% of the NADH tetrazolium reductase activity and 50% of the NADPH tetrazolium reductase activity is extracted. The solubilized protein-detergent complexes are separated on a polyacrylamide gel under non-denaturing conditions. Remnant membrane fragments do not penetrate the gel. After electrophoresis, gels have been stained for NAD(P)H dehydrogenase activity (Fig. 4A,B). It was observed that staining with NADH was faster compared to staining with NADPH. Furthermore membrane proteins from N_2-fixing cells stained more intensively than membrane proteins from NH_4Ac-grown cells. The position on the gel of the NADH tetrazolium reductase complexes originating from N_2-fixing cells and complexes originating from NH_4Ac-grown cells are identical (Fig. 4A). For the NADPH tetrazolium reductase, one band in membranes from N_2-fixing cells stains at least ten times faster than the same band in membrane isolated from NH_4Ac grown cells (arrow, Fig. 4B). The difference in activity is obvious already 1 hour after derepression of nitrogenase activity. This band with induced NADPH tetrazolium reductase was extracted from the gel and also the band of the control lane with membrane proteins from cells grown in the presence of NH_4Ac. The polypeptides present in both gel extracts were analyzed on SDS-polyacrylamide gels (Fig. 4C). As observed by others [22] the dehydrogenase complexes contain many polypeptides. The pattern is identical for the two modes of growth, except for a polypeptide of M_r 29000, that is only detectable in the NADPH dehydrogenase complex extracted from membranes isolated from cells grown N_2-fixing (see the arrow in Fig. 4C). The presence of the 29 kDa polypeptide has been shown to be reproducible in four separate experiments.

5.5 DISCUSSION

In this paper a relationship has been demonstrated between the rate of electron transfer through the respiratory chain and the whole cell nitrogenase activity. Replotting data obtained by others [23,24] also shows a similar linear relationship between these activities in Azotobacter cells.

This phenomenon can be explained by the following possibilities: (a) an increase in the rate of respiration of cells results in an increased proton motive force, giving an increase in the membrane potential and/or the ATP/ADP ratio; (b) the concentrations of the enzymes needed for whole cell N_2-fixation (electron transport and nitrogenase) are higher in cells with a higher respiration rate; (c) when cells have a higher rate of respiration the catalytic activity of the electron transport chain to nitrogenase is enhanced. Explanation (a) is probably only true for cells grown under a severe oxygen limitation. At higher oxygen input rates, it is known that respiration in A.vinelandii is uncoupled [25]. The proton motive force is kept at a constant level by varying the degree of uncoupling. It has been shown that the ATP/ADP ratio in A.vinelandii cells is fairly independent of the growth conditions [4,5,16]. For these reasons we do not expect the maximal membrane potential to be significantly different in different types of cells. Under our experimental conditions, at maximal nitrogenase activities the membrane potential had a maximum value and did not increase at higher oxygen input rates. Explanation (b) is excluded by the experiment shown in Table 1, where the same cells were incubated with different carbon sources and give higher activities without any adaptation. Additional evidence can be found in the literature [4], where it has been shown for one batch of cells that inhibition of the rate of respiration caused inhibition of nitrogenase activity, without an effect on the ATP/ADP ratio. In our opinion explanation (c) is most likely. In a previous paper we showed, that the catalytic activity of nitrogenase in vivo is determined by the rate of electron transport to nitrogenase [12]. In addition, we propose that the rate of electron transport to nitrogenase in A.vinelandii is determined by the rate of electron transfer to oxygen in the respiratory chain. The nature of the interaction between both processes is still unknown, but there may be further analogy with solute transport systems as studied by the group of Konings [10,11].

From the derepression experiments no clear answer was obtained for the induction of an electron transport chain as observed in Klebsiella pneumoniae [26]. In contrast to K.pneumoniae induction of nitrogenase activity in A.vinelandii is much faster. 5 min after transfer to nitrogen-free medium, synthesis of the nitrogenase polypeptides is detectable and nitrogenase activity after 20 min. Apparently the period between 5 and 20 min is

necessary for processing the nitrogenase proteins and/or incorporation of (Mo)FeS clusters. With respect to proteins possibly involved in electron transport to nitrogenase, only the synthesis of a limited number of proteins were detected: flavodoxin II, most likely the physiological reductant for nitrogenase under the derepression conditions used and two polypeptides of M_r 29000 and 30000, present in the cytoplasmic membrane. It should be noted, that our experiments do not definitely prove, that the 29 kDa and 30 kDa membrane proteins are involved in the process of nitrogen fixation. They also may arise from the N-starved situation that occurs during derepression. Besides synthesis of two membrane polypeptides, a NADPH-nitrobluetetrazolium reductase activity becomes detectable during derepression. This induced activity was associated with the presence of a polypeptide of M_r 29000 in a solubilized NADPH dehydrogenase complex. Whether this 29 kDa polypeptide is identical to the 29 kDa polypeptide that was radioactively labelled during the derepression remains to be elucidated. It is remarkable, that the NADPH dehydrogenase activity induced during derepression has not been detected by measurements with intact membranes, but only after electrophoresis. However from Fig. 1 it can be deduced, that the rate of electron transfer to nitrogenase is only 7% of the rate of electron transfer to oxygen. It is therefore difficult, with a general dehydrogenase assay, to detect the induction of a specific NADPH dehydrogenase involved in electron transport to nitrogenase in the presence of other more active NADPH dehydrogenases. But solubilization of membranes with triton X-100 followed by electrophoresis at pH 8.8, might have separated the NADPH dehydrogenase complex involved in reduction of flavodoxin II from the other membrane-bound NADPH dehydrogenases and made the detection of this activity possible.

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6. GENERAL DISCUSSION

6.1 The activity of nitrogenase in vivo

Nitrogenase needs ATP and a strong reductant for activity (E_h<-460 mV). How ATP is generated in living cells is sufficiently known. Less is known about the generation of the strong reductant for nitrogenase. In fermenting bacteria reducing power is mainly provided by the thioclastic reaction (eq. 2 in Chapter 1). In extracts from these bacteria nitrogenase activity can be measured with pyruvate or dithionite as electron donor. This activity is equal to the whole cell activity. In all other nitrogen-fixing organisms the activity of the thioclastic reaction is too low to be significant for the generation of reductant for nitrogenase [9]. In aerobic bacteria pyruvate: ferredoxin oxidoreductase activity (the thioclastic reaction) is absent [12]. These bacteria have a pyruvate dehydrogenase complex reducing NAD^+ (E_m = -320 mV) instead of ferredoxin. How reducing equivalents for nitrogenase are generated in obligate aerobes is virtually unknown even after ca. 20 years of research. The reason is that after breakage of cells the physiological electron transport system is totally inactive or at most 5% of the dithionite activity [3]. All information about the electron transport chain to nitrogenase is derived from experiments with whole cells.

The research reported in this thesis focussed on the electron transport to nitrogenase in the obligate aerobic free-living bacterium Azotobacter vinelandii as did three earlier thesises from this laboratory [7,14,25]. A relatively new immunological method was used to measure the catalytic activity of the enzyme nitrogenase in intact cells (Chapter 3). It appeared that the nitrogenase activity in vivo was very high. Sometimes even twice the activity measured in vitro with an ATP regenerating system and dithionite as electron donor. It is unlikely that in vivo the ATP supply is more active than the ATP regenerating system used in vitro. Apparently the electron transport system to nitrogenase in vivo is more effective than the in vitro donor dithionite. It was also observed that the catalytic activity of nitrogenase in intact cells increased when the growth rate of cells increased. This increase was not due to alterations in the enzyme nitroge-

nase itself but due to an increase in electron transport and/or ATP supply. The ATP supply being the limiting factor for whole cell nitrogenase activity can be excluded by the observation that at conditions where nitrogenase activity is maximal, the internal ATP/ADP ratio in Azotobacter cells is fairly constant and independent of the growth conditions [7,13]. Therefore it can be concluded, that nitrogenase activity in intact A.vinelandii cells is mainly determined by the rate of electron transport to nitrogenase.

Also in in vitro experiments, the generation of reductant for nitrogenase is important for the overall activity. Using purified nitrogenase from Klebsiella pneumoniae and the artificial electron donor $Na_2S_2O_4$ a detailed kinetic mechanism of nitrogenase action was developed [16,27]. An important feature of the model is, that the rate-limiting step in nitrogenase catalysis is the dissociation of oxidized Fe protein from reduced MoFe protein after MgATP-induced electron transfer has occurred between these proteins. The oxidized Fe protein cannot be reduced when bound to MoFe protein. After dissociation, oxidized Fe protein must be reduced rapidly, because of an association of oxidized Fe protein with MoFe protein will cause inhibition of nitrogenase [27-29]. In Chapter 3 it has been shown, that the concentration of the nitrogenase proteins is high in A.vinelandii. But no indications were found that nitrogenase was inhibited, since the catalytic activity of nitrogenase in vivo was higher than in vitro. This observation might mean that in vivo the concentration of oxidized Fe protein is kept very low, because of a very efficient electron donating system to the Fe protein. It is also possible that in vivo the association and dissociation constants of Fe protein and MoFe protein are altered [29]. A third possibility is, that the physiological electron donor reduces Fe protein without the need of dissociation from MoFe protein. In my opinion the third explanation is not unlikely. The electron donor required for nitrogenase activity must have a low redox potential and is therefore very reactive. It is known for example that flavodoxin hydroquinone in solution gives rise to spontaneous H_2 evolution. Generation of reducing equivalents for nitrogenase in a tight enzyme complex would have the advantage of preventing reactions of the reduced low potential electron carriers with proteins involved in other metabolic pathways as for instance respiration.

6.2 Electron transport and the membrane potential

As reviewed in Chapter 1 the proton motive force, especially the membrane potential is involved in electron transport to nitrogenase in aerobic nitrogen-fixers. For Azotobacter vinelandii it was shown that NH_4^+ rapidly inhibited nitrogenase activity by specifically switching off the flow of reducing equivalents to nitrogenase by lowering the $\Delta\Psi$ [13]. This observation was questioned by others, since they found that there was no short-term inhibition of nitrogenase activity in logarithmic growing cultures [6]. In chapter 2 the discrepancies in the literature are resolved by showing that the extent of inhibition of nitrogenase activity by NH_4Cl is variable and depends on the growth and test conditions. How to explain the short-term effect of NH_4^+ on a molecular level? Why causes NH_4^+ under certain conditions a strong inhibition of nitrogenase activity and under other conditions hardly any? To understand this, experiments have been performed with a tetraphenylphosphonium specific electrode to measure the membrane potential [15]. What happened, when NH_4Cl was added to cells can be summarized as follows: (a) For cells incubated under energy limiting conditions, addition of NH_4^+ caused a decrease in $\Delta\Psi$ and therefore inhibited electron transport to nitrogenase. Under these conditions the experiments of Laane et al. [13] were performed. (b) For cells with a high respiration rate and incubated under optimal conditions for N_2-fixation, addition of NH_4^+ had no effect, neither on the membrane potential nor on the nitrogenase activity. This is also observed by Barnes and Zimniak [2]. (c) When cells were incubated at oxygen concentrations, inhibitory for N_2-fixation, NH_4^+ inhibited nitrogenase activity strongly, whereas the $\Delta\Psi$ remained constant. How to explain the inhibition of nitrogenase under conditions where NH_4^+ has no effect on $\Delta\Psi$? For A.vinelandii no experiments about this phenomenon are reported, but for Rhodopseudomonas sphaeroides there is more clarity. When this organism was incubated under conditions of excess energy, NH_4^+ caused a strong inhibition of nitrogenase activity, but had no effect on $\Delta\Psi$ [8]. When glutamine synthetase was inhibited with methionine sulfoximine (MSX), added NH_4^+ was taken up by cells, but was not assimilated. It was observed that NH_4^+ did not inhibit nitrogenase under these conditions [8]. Measurements of intracellular glutamine pools and

experiments with inhibitors of glutamate synthase indicated, that the assimilation product of NH_4^+, that inhibits nitrogenase activity, is most likely glutamine [1,17,18,26,31]. Because glutamine is not an inhibitor of the enzyme nitrogenase, the most likely mechanism of inhibiting nitrogenase is inhibition of electron transport to nitrogenase. Although conditions are not specified, a relief of NH_4^+ inhibition by addition of MSX has been described for Azotobacter chroococcum [4], suggesting regulation of nitrogenase activity by the glutamine pool in Azotobacter species too. Conclusively the short-term inhibition of nitrogenase activity by NH_4^+ shows, that electron transport to nitrogenase in Azotobacter is regulated by the membrane potential ($\Delta\Psi$) and possibly by the intracellular concentration of glutamine.

6.3 Electron transport and respiration

In obligate aerobic nitrogen-fixers the respiratory chain is involved in the process of N_2-fixation in at least three ways: (a) to protect the oxygen-sensitive nitrogenase the interior of cells is kept at a low free oxygen concentration by a high respiration rate, (b) ATP is generated by oxidative phosphorylation, (c) the generation of reducing equivalents for nitrogenase requires a high membrane potential. This membrane potential is generated by electron transfer through the respiratory chain.

In Chapter 5, experiments indicate a more direct interaction between electron transport through the respiratory chain and electron transport to nitrogenase. The important observation was that a change in the rate of respiration of cells always caused a similar change in nitrogenase activity even under conditions where the ATP/ADP ratio and $\Delta\Psi$ were assumed to be constant. Such a relationship would also clarify the oxygen shock experiments shown in Chapter 3. When cells are exposed to excess oxygen nitrogenase activity is immediately switched-off. The reason for this immediate switch-off is not known. A possible explanation is given by Scherings [25], who states that the electron donor flavodoxin is oxidized. In the next hours the cells respond by increasing the respiration rate, and after some

time growth restarts. To prevent inhibition of the respiration rate by the proton motive force respiration must be partly uncoupled. It is unlikely to expect significant increases in the ATP/ADP ratio by increased respiration. Still a more than twofold increase in nitrogenase activity was measured after an O_2-shock. In the experiments shown in Chapter 3 after an O_2-shock the respiration rate of cells was increased by a factor 2.2 and the nitrogenase activity by a factor 2.5. This observation is in accordance with a proposed relationship between electron transport to nitrogenase and electron transfer activity in the respiratory chain. This interaction is possibly not restricted to A.vinelandii. From the literature such a relationship can be proposed for soybean bacteroids [23,24] and Anabaena species, when fixing nitrogen in the dark [11,22].

6.4 Proteins involved in electron transport to nitrogenase

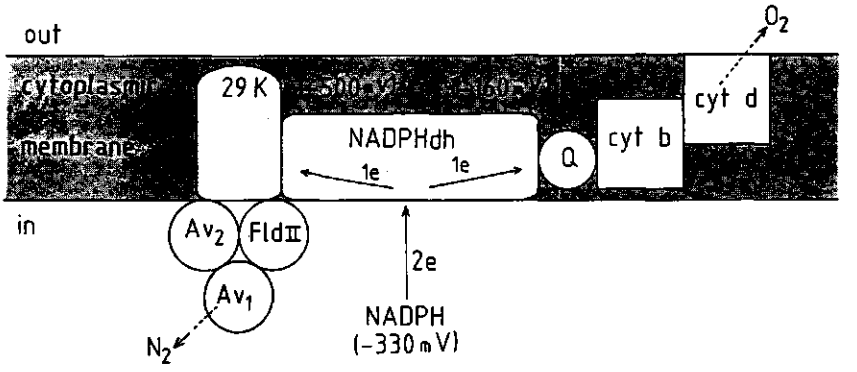
As reviewed by Scherings [25] biochemical data point to flavodoxin as the ultimate reductant for nitrogenase in Azotobacter species. For instance in Chapter 3, we showed that flavodoxin is a better electron donor for nitrogenase in extracts than dithionite. Flavodoxin makes Fe protein function more efficiently, since less Fe protein is necessary to saturate MoFe protein. With flavodoxin, nitrogenase activities in extracts approximate whole cell activities. In Chapter 4, the hypothesis of flavodoxin being the electron donor for nitrogenase was supported by physiological experiments. But it was also shown that at least three different flavodoxins can be isolated from A.vinelandii. Complete separation of the three flavodoxins was achieved by Fast Protein Liquid Chromatography. Since the presence of different flavodoxins in Azotobacter has not been reported before it is possible that a lot of earlier data on Azotobacter flavodoxin have been obtained with mixtures of flavodoxins I and II. We have shown, that only flavodoxin II is involved in N_2-fixation. Its synthesis after derepression parallels synthesis of the nitrogenase proteins (Chapters 4 and 5) and its concentration is tenfold higher in N_2-fixing cells compared to cells grown on NH_4Ac (Chapter 4). Photochemically reduced flavodoxin I is not oxidized by nitrogenase, while flavodoxin II is. No significant synthesis of ferre-

doxin or flavodoxin I have been detected during derepression. However one should be careful in drawing the conclusion that only flavodoxin II and not ferredoxin I is involved in electron transport to nitrogenase. The Burk's medium [21] used to grow A.vinelandii is always turbid. Recent analysis in our laboratory showed that most of the iron in the medium is precipitated. Consequently, although enough iron is added to the medium most of it is not readily available to the cells. Especially at high growth rates there can be a shortage of iron. This makes the statement of flavodoxin being a constitutive protein in Azotobacter species less firm [30]. Maybe as in other organisms flavodoxin replaces ferredoxin as electron donor during iron-deficient growth. This would at least explain why in our cultures large differences were found in the cellular concentration of flavodoxin (Chapters 3 and 4). The amount of soluble iron in the medium would have determined to what extent ferredoxin and flavodoxin were synthesized. Especially in chemostat cultures the amount of iron in the culture vessel might have been low. A way to prevent precipitation of the iron is additon of citrate to Burk's medium. Strikingly two years ago people working on ferredoxin I from A.vinelandii switched over from growth of cells on Burk's medium to growth on medium containing citrate [19,20]. The reason might have been, that such cells give higher yields of ferredoxin.

As described in Chapter 5, except for flavodoxin, no soluble proteins involved in electron transport to nitrogenase could be detected during derepression. Proteins that might play a role in this process are two membrane bound polypeptides of M_r 29000 and 30000 and a membrane bound NADPH dehydrogenase. However it might well be that the electron transport system to nitrogenase is not at all nif specific. In cells grown on NH_4^+, flavodoxin II is present and in addition to that, NH_4^+-grown cells contain flavodoxin III which has a similar redox potential as flavodoxin II. It is very well possible that cells grown on NH_4^+ and N_2-fixing cells make use of the same flavodoxin reducing system. In that case genetic identification of the flavodoxin reducing system will be difficult, because a mutation in the electron transport pathway to nitrogenase might also be lethal for cells grown on NH_4^+, making it impossible to grow up such mutants.

6.5 Proposal for electron transport to nitrogenase

Schemes for electron transport to nitrogenase have been proposed by Benemann, Haaker (Chapter 1), Scherings [25] and Laane [14]. As long as none of the schemes can be proven by in vitro experiments there is no need for proposing again another model. The next figure must therefore be considered as a summary of some new ideas on the electron transfer chain to nitrogenase. It is not my intention to present it as the only correct proposal.



In the scheme an NADPH dehydrogenase in the cytoplasmic membrane is reduced by 2 electrons from NADPH (E_h= -330 mV). The idea is that the NADPH dehydrogenase is specifically oxidized. One electron (E_h~-160 mV) is donated to a respiratory chain component at the redox level of ubiquinone, oxygen being the ultimate acceptor. The other electron can be transferred to a redox center with a low potential (E_h~-500 mV) and this center can reduce flavodoxin III in cells grown on NH_4^+ or flavodoxin II (and ultimately N_2) in N_2-fixing cells. It is postulated that the 29-kDa polypeptide plays a role in the latter process.

As was made credible for cyanobacteria (Chapter 1) in this scheme the membrane potential is no longer the driving force for reversed electron flow to nitrogenase. As proposed by Davis and Kotake [5] $\Delta\Psi$ might have a function in the regulation of the Mg^{2+} concentration in the cytoplasm. Another possibility is that the membrane potential plays a role in the formation of an enzyme complex on the cytoplasmic membrane from the soluble flavodoxin and nitrogenase proteins. This agrees with the observation that

in vivo nitrogenase is bound to the membrane [10]. An enzyme complex has the advantage that electrons with a low redox potential are not wasted by unwanted side reactions.

The proposed model also explains oxygen inhibition of nitrogenase. At high concentrations of oxygen the components of the respiratory chain and all redox centers in the NADPH dehydrogenase are oxidized. All electrons are chanelled away from flavodoxin II to oxygen.

In Chapter 5 it was demonstrated that electron transport to oxygen mediated by the membrane bound NADPH dehydrogenase was inhibited by NAD^+. The NAPDH dehydrogenase activity measured with nitroblue tetrazolium as electron acceptor was not inhibited by NAD^+. Since the concentration of NAD^+ is high in *A.vinelandii* cells, it is unlikely that the membrane bound NADPH dehydrogenase has a physiological role as electron donor to the respiratory chain, although it can function as electron donor *in vitro*. The scheme summarizes the mechanism of action of the NADPH dehydrogenase. It reduces flavodoxin, and for this activity electron transport to oxygen is obligatory. This last aspect would explain why until now no satisfactory rates for nitrogenase activity with physiological substrates have been measured *in vitro* even in the presence of an artificially generated $\Delta\Psi$. *In vitro* nitrogenase activity is always measured anaerobically. While the NADPH:flavodoxin oxidoreductase can only function in the presence of an active respiratory chain. In my opinion, it is therefore impossible to mimic the *in vivo* situation *in vitro*, because *in vivo* oxygen is present at the outside of the cell and the anaerobic environment needed for nitrogenase is inside the cell. Aerobic experiments *in vitro* make no sense, because even if flavodoxin is reduced it will be immediately oxidized by oxygen. Probably the only way to detect the enzyme activity is making use of an irreversible reduction reaction like tetrazolium reduction described in Chapter 5.

The last aspect of the model is, that it gives a very simple explanation for the tight coupling between electron transfer activity in the respiratory chain and nitrogenase activity (Chapter 5). Since for each electron used in N_2-fixation at least one electron has to be oxidized by the respiratory chain.

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SUMMARY

The enzyme nitrogenase requires MgATP, an anaerobic environment and an electron donor with a low redox potential for activity. The experiments described in this thesis deal with the electron transport to nitrogenase in Azotobacter vinelandii. It has been shown previously that the flow of reducing equivalents to nitrogenase is regulated by the $\Delta\Psi$ component of the proton motive force. Short-term inhibition of nitrogenase activity by externally added NH_4^+ would be caused by lowering the $\Delta\Psi$ [1]. In Chapter 2 it has been shown that the extent of inhibition by NH_4Cl is variable and depends upon the incubation conditions of the cells. Conditions are described, where nitrogenase activity is hardly inhibited by addition of NH_4Cl and also conditions, where uptake of NH_4Cl results in complete inhibition of nitrogenase activity. These results are discussed in Chapter 6. In addition to the membrane potential glutamine is proposed as another regulator of electron transport to nitrogenase.

In Chapter 3 it has been shown, that whole cell nitrogenase activity is determined by the generation of reducing equivalents for nitrogenase. The physiological electron transport system to nitrogenase is very effective compared to the electron donor dithionite often used in in vitro experiments. It has been shown that whole cell nitrogenase activity in vivo can be twice the activity measured in vitro. The consequences of this finding are discussed with respect to the mechanism for nitrogenase catalysis in vitro and in vivo.

In Chapter 4 it has been shown, that three different flavodoxins can be isolated from A.vinelandii cells. Experimental evidence indicates that only flavodoxin II is involved in N_2-fixation. The concentration of flavodoxin II is tenfold higher in N_2-fixing cells compared to cells grown on NH_4Ac. And its synthesis seems to be under the same regulatory control as the nitrogenase proteins.

In Chapter 5 evidence is presented that a membrane bound NADPH dehydrogenase and two membrane bound polypeptides of relative molecular mass 29000 and 30000 probably play a role in electron transport to nitrogenase. Furthermore in Chapter 5 it has been demonstrated that there is a linear relationship between nitrogenase activity and the rate of respira-

tion of A.vinelandii cells. It is proposed that the generation of reducing equivalents for nitrogenase is directly controlled by electron transfer activity in the respiratory chain.

In Chapter 6 the new findings on the electron transport to nitrogenase are summarized in a scheme. In the scheme the electron carrier flavodoxin II is reduced by a membrane bound NADPH dehydrogenase only when the respiratory chain is functioning.

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SAMENVATTING

Naast water, zonlicht, fosfaat en kalium-zouten, is stikstof één van de stoffen, die planten nodig hebben voor de groei. Heel vaak is de kleine hoeveelheid bruikbare stikstof in de bodem de beperkende factor voor de groei van gewassen. Hoewel 80% van de aardse atmosfeer uit stikstof bestaat, kan het in deze vorm (N_2) niet benut worden door planten. Eerst moet stikstof uit de lucht omgezet worden in ammonium (NH_4^+). Dit proces (stikstof binding) wordt alleen uitgevoerd door een beperkt aantal bacteriën. Bacteriën zijn eencellige organismen met een lengte van 0,001 mm of minder. In de natuur zijn stikstofbindende bacteriën essentiëel bij het in stand houden van de vruchtbaarheid van de bodem. Het meest bekend zijn de bacteriën die zich bevinden in de wortelknolletjes van vlinderbloemige planten zoals erwten, bonen, soja, klaver, wikke en lupine. Naast de biologische stikstofbinding wordt sedert 1930 ammonium op grote schaal bereid uit aardgas in de stikstofmeststoffen industrie. De industriële stikstofbinding bedraagt ongeveer 15% van de totale stikstofbinding op aarde. Intensieve landbouw is welhaast ondenkbaar geworden zonder het gebruik van (stikstof)kunstmest.

Uitbreiding van de wereldvoedselproductie kan in principe door het op grotere schaal toepassen van kunstmest. Een oplossing die echter voor veel landen te duur is. Bovendien is het aardgas, dat nodig is voor het maken van de ammonium geen onuitputtelijke grondstof. Geen wonder dat juist na de energiecrisis van 1973 de belangstelling voor de biologische stikstofbinding is toegenomen. Op dit moment doen meer dan 1000 wetenschappers uit 60 verschillende landen onderzoek aan de biologische stikstofbinding. Een vanuit wetenschappelijk oogpunt interessante vraag daarbij is: hoe komt het toch dat alleen bacteriën stikstof uit de lucht kunnen binden tot ammonium? Waarom doen planten dit niet zelf? En zou het misschien mogelijk zijn om een plant zo te veranderen, dat zij in staat is om haar eigen stikstof te binden? Dit laatste zou dan de stikstofbemesting overbodig maken.

Moleculair-biologen hebben inmiddels de erfelijke eigenschap opgespoord die zorgt voor de stikstofbinding in bacteriën. Ook is het al mogelijk gebleken deze erfelijke eigenschap over te brengen in planten. Maar het

stikstofbindende systeem blijkt dan niet te werken. Waarom het systeem dan niet werkt en wat er voor nodig is om het systeem wel te laten werken, zijn vragen waar de biochemie zich mee bezig houdt. Uit biochemisch onderzoek is 20 jaar geleden gebleken, dat het stikstofbindende systeem in wezen bestaat uit twee eiwitten. Deze twee eiwitten vormen samen een enzym: het nitrogenase. Wanneer het nitrogenase enzym in een reageerbuis gestopt wordt met twee chemische stoffen (dithioniet en MgATP) wordt stikstof uit de lucht omgezet in ammonium. Dithioniet levert de electronen voor deze reactie en MgATP de energie. De reactie verloopt alleen, wanneer zuurstof niet aanwezig is (of zoals dat heet onder anaerobe omstandigheden). Uit de biochemische eigenschappen blijkt dat voor stikstofbinding in een levende bacterie-cel in ieder geval 4 dingen nodig zijn:

1) de erfelijke eigenschap voor het enzym nitrogenase,
2) een systeem dat dezelfde functie heeft als dithioniet,
3) een systeem dat MgATP maakt,
4) een zuurstof vrije omgeving voor het enzym.

Zoals gezegd, is over punt 1 voldoende bekend om de stikstofbindingseigenschap over te kunnen brengen van het ene levende wezen naar het andere. Echter wil nitrogenase werken dan is het nodig, dat ook aan voorwaarden 2,3,4 voldaan wordt.

Het maken van MgATP (punt 3) zal niet het grootste probleem zijn, omdat dit proces in alle levende dieren en planten gebeurt en dus niet iets speciaals is voor stikstofbinding. Wel kan hierbij opgemerkt worden, dat biochemisch onderzoek heeft aangetoond, dat nitrogenase voor de omzetting van stikstof in ammonium veel MgATP nodig heeft. Het maken van MgATP kost energie. Mocht het ooit lukken een plant haar eigen stikstof te laten binden, zal dit dus energie kosten voor de plant. Deze energie kan dan niet gebruikt worden voor groei. Bovendien moet bedacht worden dat nitrogenase een eiwit is en iedereen weet dat eiwit "rijk is aan energie". Voor het maken van het enzym nitrogenase is energie nodig. Aangezien in dit proefschrift gebleken is, dat 10% van het eiwit van een stikstofbindende bacterie uit nitrogenase bestaat, gaat het om een niet te verwaarlozen hoeveelheid. Behalve voor het maken van MgATP zal een plant dus ook nog eens energie moeten stoppen in het maken van het enzym nitrogenase. Een

stikstofbindende plant is misschien wel ideaal vanuit het oogpunt van bemesting, maar niet vanuit het oogpunt van groeisnelheid en opbrengst. Zeer waarschijnlijk zullen stikstofbindende planten langzamer groeien, dan hun soortgenoten die kunstmatig bemest worden.
Het verschijnsel dat nitrogenase alleen werkzaam is in een zuurstof vrije omgeving (punt 4) vraagt om speciale voorzieningen. Voor sommige bacteriën is dit geen probleem, omdat ze leven in een zuurstofvrij milieu (bijvoorbeeld slootmodder). De bacterie Azotobacter vinelandii, waar ik onderzoek aan gedaan heb, leeft echter gewoon in lucht (20% zuurstof). De Azotobacter bacterie heeft in zijn celwand een mechanisme zitten dat voor de ademhaling zorgt (ademhalingsketen). Deze ademhalingsketen ademt alle zuurstof uit de oplossing, waarin de bacterie groeit (=groeimedium) weg. Hierdoor is binnen in de bacterie-cel, waar het nitrogenase zit, geen zuurstof aanwezig. Wil stikstofbinding in planten plaatsvinden, dan zal dat op een plaats moeten gebeuren waar geen zuurstof aanwezig is of waar alle zuurstof verademt wordt. De groene delen van een plant lijken hierdoor al een minder geschikte plaats voor stikstofbinding, omdat in de groene delen onder invloed van zonlicht meer zuurstof vrijkomt dan dat er wordt verbruikt.

Het onderzoek, beschreven in dit proefschrift, heeft zich voornamelijk gericht op punt 2: welk systeem in de bacterie-cel heeft dezelfde functie als dithioniet? Dithioniet is namelijk een chemische stof, die niet in de bacterie voorkomt. Een van de dingen, die ik gevonden heb, is dat het "dithioniet" systeem (punt 2) heel nauw gekoppeld is aan de ademhaling van de bacterie (punt 4). Om nitrogenase te kunnen laten werken verademt de bacterie alle zuurstof uit het groeimedium (punt 4). Bij ademhaling komt energie vrij en deze energie wordt gebruikt door het systeem, wat in de bacterie de rol van dithioniet vervult (punt 2). Hoe sneller de ademhaling door de bacterie, hoe beter het "dithioniet" systeem (punt 2) en hoe hoger de snelheid waarmee stikstof gebonden wordt. Als hulpmiddel om uit te zoeken, hoe het "dithioniet" systeem in elkaar zit, is gekeken naar het effect wat ammonium heeft op de stikstofbinding. Uit de literatuur was bekend, dat na toevoegen van ammonium aan groeimedium, de bacterie stopt met stikstof te binden. Dit is een van de voordelen van biologische stikstofbinding. Doordat het proces stopt als er voldoende ammonium aan-

wezig is in de bodem, wordt overbemesting voorkomen. Er bestond echter verschil van mening over de vraag of de stikstofbinding al na enkele minuten na toevoegen van ammonium stopt, of dat het proces nog enkele uren doorgaat. In hoofdstuk 2 van dit proefschrift laat ik zien dat beide gevallen mogelijk zijn. Als de bacterie kampt met een tekort aan energie stopt de stikstofbinding onmiddellijk na toevoegen van ammonium. Is er voldoende energie aanwezig, dan gaat de stikstof binding nog enige tijd door. Na een dag groeien op ammonium is er totaal geen stikstofbinding meer. De nitrogenase eiwitten blijken dan niet meer aanwezig te zijn in de bacterie (hoofdstuk 3). Zeer waarschijnlijk is ook het "dithioniet" systeem afwezig, omdat het voor de bacterie geen zin heeft om dit systeem te maken als nitrogenase niet werkt. Door de stikstof bindende bacterie te vergelijken met dezelfde bacterie, maar dan gegroeid op ammonium zodat deze geen stikstof bindt, heb ik één eiwit kunnen vinden, die in de levende bacterie dezelfde rol speelt als dithioniet in een reageerbuis (hoofdstuk 4). Ook heb ik bij bacteriën die op ammonium groeiden, ineens het groeimedium vervangen door een medium zonder ammonium. Het eerste wat de bacteriën dan gaan doen, is alle dingen maken die nodig zijn voor stikstofbinding. Al na 20 minuten is er stikstofbinding meetbaar. Door nu te kijken, wat er in die 20 minuten allemaal verandert in de bacterie, heb ik een ander eiwit kunnen aantonen, die mogelijk ook een onderdeel vormt van het "dithioniet" systeem (hoofdstuk 5). Het maken van beide eiwitten, waarvan ik gevonden heb dat ze onderdeel zijn van het "dithioniet" systeem wordt bepaald door twee of meer erfelijke eigenschappen. Deze erfelijke eigenschappen zullen eerst opgespoord moeten worden. Samen met de erfelijke eigenschap voor het nitrogenase (punt 1) kunnen ze dan overgebracht worden in een andere bacterie of een plant, in de hoop dat een werkzaam stikstofbindend systeem ontstaat.

Een andere vraag, die in het proefschrift beantwoord is, heeft betrekking op hoe goed nitrogenase in een levende bacterie werkt. De laatste jaren is een gedetailleerd model ontwikkeld over de werking van nitrogenase in een reageerbuis. Dit model voorspelt echter dat hoe hoger de concentratie van het enzym wordt, hoe langzamer nitrogenase stikstof gaat omzetten in ammonium. In hoofdstuk 3 laat ik zien, dat de concentratie nitrogenase in een levende bacterie wel 100x hoger is, dan de concentratie

die gebruikt wordt in reageerbuis experimenten. Volgens het model zou nitrogenase dus zeer slecht moeten werken. Het blijkt echter (hoofdstuk 3) dat het enzym in een levende bacterie 2x zo snel stikstof bindt, dan in een reageerbuis. Een en ander betekent dat het model, dat beschrijft hoe nitrogenase werkt in een reageerbuis, nog niet goed weergeeft hoe het enzym in een levende cel werkt. De oorzaak zit hem in het feit, dat in een reageerbuis altijd gewerkt wordt met de chemische stof dithioniet; terwijl een levende bacterie hier een ander systeem voor heeft wat kennelijk veel beter werkt.

CURRICULUM VITAE

Jan Klugkist werd op 2 juli 1956 te Alphen aan de Rijn geboren. In 1974 behaalde hij het diploma Atheneum-B aan de Chr. Scholengemeenschap in Katwijk en begon met de studie moleculaire wetenschappen aan de Landbouwhogeschool te Wageningen. In januari 1977 werd het kandidaatsexamen behaald en in januari 1981 het ingenieursexamen. Hoofdrichting was de Biochemie met daarbij inbegrepen een praktijkperiode van 6 maanden aan het Academisch Ziekenhuis in Utrecht. Bijvakken waren de Virologie en de Moleculaire Biologie.

Vanaf februari 1981 tot mei 1985 was hij verbonden aan het Laboratorium voor Biochemie van de Landbouwhogeschool te Wageningen, eerst als promovendus, daarna als wetenschappelijk medewerker. Vanaf 1 juni 1985 is hij in dienst van het Unilever Research Laboratorium te Vlaardingen.