Nitrogen fixation Plant Biochemistry, Biochemistry 621, December 2003

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This is hardly a polished text, but rather something of an overview, with figures I was able to filch off the web to illustrate some issues. Please read it (though you need not puzzle through any data here) and then we can more of a discussion than a lecture on Tuesday and Thursday.

Chemistry of N_2 fixation

Nitrogen fixation is actually an endothermic reaction with a)G of -7.98 kcal/mole. Nevertheless, the reaction simply does not occur non-enzymatically because of a very large activation energy, reflecting the fact that the N_2 bond is one of the more stable in nature. The stability of that bond then dictates a great deal about the biochemical catalysis, and ultimately about the biological utilization of the process, even down to the regulation of gene expression.

The process of nitrogen fixation is performed industrially by a process termed the Haber Bosch process and for which Fritz Haber received the Novel Prize in Chemistry in 1918, although vilified for his work on chemical weapons for Germany in WWI. The process involved high heat and pressure of H_2 and N_2 and catalysts which were originally osmium and uranium, but now are a molybdo-iron complex. Remarkably, the amount of NH_3 produced by this process has doubled about every six years since the late 1950's.

Overview of the process and its history

General overview of the biochemistry and the ecology of biological nitrogen fixation (BNF)

"*Nitrogenase* is a two protein component system that catalyzes the reduction of dinitrogen to ammonia coupled to the hydrolysis of ATP. Rather remarkably, even after 35 years of study, the overall reaction stoichiometry is still not unambiguously determined. The uncertainties are expressed in the following equation for the overall enzyme reaction (from Curr Opin Chem Biol.4:559 (2000)):

$$N_2 + (6+2n)H^+ + (6+2n)e^- + p(6+2n)ATP \longrightarrow 2NH_3 + nH_2 + p(6+2n)ADP + p(6+2n)P_i"$$

Because the N-N bond is so stable, the electrons that are added must be of extremely low potential. The vast majority of nitrogen-fixing organisms solve this by starting with the low potential electrons found on ferredoxin or flavodoxin and transferring a single electron to the a terminal reductase for nitrogenase fixation, which is termed *dinitrogenase reductase*, the Fe protein, component II or NifH (Fig. 1). This protein dimer carries a Fe_4S_4 metal center which is the element reduced by the added electron. Dinitrogenase reductase then hydrolyzes ATP in transferring one electron to the protein that actually fixes N_2. This protein is termed *dinitrogenase*, the MoFe protein, component I or NifKD. It is an "$_2$$_2$ tetramer with two pairs of distinct metal clusters: one pair of Fe_8S_7 clusters and one pair of unique $MoFe_7S_9$ cluster termed FeMo-co. Oxidized dinitrogenase reductase decouples from dinitrogenase and releases the ADP, is re-reduced, binds ATP and again transfers an electron to dinitrogenase. When dinitrogenase is reduced with 8 electrons it rapidly transfers these electrons, as well as 6 protons, the a bound N_2,

producing 2 molecules of NH_3. The other two electrons, together with two additional proteins, are combined to form one molecule of H_2. Oddly enough, this hydrogenase activity is obligate for the enzyme and cannot be eliminated even under high pressures of N_2.

Rather surprisingly, given the importance of this reaction in biology, the vast majority of nitrogen-fixing organisms, termed diazotrophs, all use one or more protein systems homologous to that described above. Almost all of these have the system described, termed the molybdenum system, but some have one or even two additional systems. In one of these, the Mo atom is replaced by vanadium, and the enzyme seems to be slightly less efficient. In the other system the Mo is replaced by another iron atom, and these are termed Fe-only nitrogenases. Interestingly, these genes clusters for these "alternate" nitrogenases have roughly a complete set of genes for the process, which are mostly homologous with those of the Mo nitrogenase. Organisms typically employ the Mo nitrogenase if there is sufficient molybdenum available, but then use the Va or Fe systems if necessary.

Remarkably, given the huge number of man-years devoted to nitrogen fixation and its biochemistry, a completely different nitrogenase was discovered in 1997 by Ortwin Meyer that bear no similarity to the above systems and will be discussed below.

Irrespective of the specific enzyme system, nitrogenases are slow, with turnover rates of several per second. This is a problem for an enzyme being called upon for producing all of the fixed nitrogen in the cell. This then has the implication that nitrogenase must be abundant if a cell is to grow on N_2. The second commonality is that the process is always energy-intensive. As a consequence, N_2 is the more energetically poor nitrogen source imaginable. It is therefore used only when the cell is starving for fixed nitrogen and no other sources are available. Finally, the enzymes (other than the Meyer enzyme, which is O_2-stable) are among the most oxygen-labile known. As a consequence, cells either need to create biochemical mechanisms for protecting the nitrogenase from oxygen, or else only perform the process under anoxic conditions.

Role in the real world: symbiotic and non-symbiotic

The atmosphere has 4×10^{21} gm of N, soil has 3×10^{17} gm, and rocks have 2×10^{23} gm; NO_3 and NH_3 (the only forms available to plants and most microbes) together are at $2x\ 10^{16}$ gm in soil and 1.2×10^{16} gm N is found in plants. Industrial N_2 fixation is 8×10^{13} gm/yr and biological is about the same, though denitrification by prokaryotes returns about 1.2×10^{14} gm/yr to the atmosphere, primarily as N_2. In the oceans, the most important nitrogen fixer is apparently a filamentous (non-heterocystous) cyanobacterium named *Trichodesmium*, which is discussed a bit later.

Agronomically, nitrogen fixation is primarily important to the growth of legumes, many of which form very specific symbioses with specific bacteria, generically termed rhizobia. There are other less specific interactions, termed associative interactions, discussed later. In both cases, the bacteria can supply substantial levels of fixed to the plants, though plant growth is typically not as fast as with high levels of chemical fertilizer. As a consequence, in many crops in the developed world, where yield rather than cost efficiency is the goal, nitrogen fixation is of only modest economic importance. Indeed, when chemical fertilizers are applied to crops that can fix N_2 the level of fixation decreases because of a variety of biological control mechanisms that sense the availability of a cheap fixed N source. Efforts to increase the economic impact by enhancing the level of nitrogenase have been unsuccessful, presumably for physiological reasons. It also seems unlikely that completely new symbiotic relationships with cereal crops, for

example, are likely to be developed. The major potential avenue for improved agronomic is probably the improvement of existing, and the development of new, associative interactions, because these are substantially less specific and therefore more amenable to manipulation (discussed later).

History of the field, with a focus on Madison:

Boussingault reported nitrogen fixation by leguminous plants in 1838, but this was disputed by Liebig, who argued that the plants obtained NH_3 from the atmosphere instead. Gilbert, Lawes and Pugh did very careful experiments at Rothamsted in Great Britain, that showed nitrogen fixation did not occur in soils that had been sterilized, but they didn't guess at the problem this treatment caused. In 1888 Hellriegal and Wilfarth reported nitrogen fixation in peas, but moreover that this was dependent on the presence of root nodules with bacteria. The work was repeated by the Rothamsted group and the matter was laid to rest.

The first paper published at UW on the topic was in about 1906, but research became more central in 1913 with E. B. Fred arrived in Agricultural Bacteriology from Virginia Tech. Fred set up a collaboration with Peterson in Agricultural Chemistry fairly early on a variety of topics, with nitrogen fixation (Fred-Peterson). Over the next 15 years, he recruited Ira Baldwin Elizabeth McCoy and Perry Wilson to become faculty members in Agricultural Bacteriology and biological nitrogen fixation remained central to the department for a number of year. Fred, Baldwin and McCoy published an influential book on the subject in 1932. Perry Wilson obtained a grant from the Fraasch foundation for $10,000 year, a vast sum, and set up labs in the Soils building, beginning work on the biochemistry of nitrogen fixation. Some of Wilson's important conclusions were that nitrogen fixation in clover was sensitive to excessive O_2 levels and that it was inhibited by H_2, though the mechanism was completely unknown for years. One of his students was Bob Burris, who was hired in Agricultural Chemistry to replace Tottingham when the latter died in 1941. There was some urgency because they needed to complete the ongoing course, Plant Biochemistry. Burris' research concentrated on the enzymology of nitrogen fixation for many years and he was also chair of Biochemistry for a quite a time, stepping down when Hector DeLuca first became chair.

A major breakthrough occurred in 1960, when Carnahan *et al.* showed consistent N_2 fixation in extracts of *Clostridium pasteurianum*. Subsequently groups in Australia, Britain and Madison purified and characterized nitrogenases from a variety of bacteria.

One of Burris' students was Paul Ludden, who subsequently was hired in Biochemistry in 1982 and only recently left in 2002. In about 1965, Bacteriology hired Winston Brill, who worked on nitrogen metabolism and was placed in a lab next to Perry Wilson's. When Wilson suffered a stroke, Brill helped Wilson's students and eventually moved into the field. I came here as a post-doc with Brill in 1975, then went to Merck and returned to take Brill's position when he left the University to run Agracetus in Middleton. Jo Handelsman was one of Brill's last students and was eventually hired in Plant Pathology. She worked on symbiotic nitrogen fixation for a number of years but has moved to environmental microbiology. Finally, Eric Triplett, who used to give these lectures until he recently decided to move to University of Florida, was a post-doctoral with Ludden and was hired in Horticulture in about 1985, where he too worked on symbiotic nitrogen fixation until quite recently. He also moved into environmental microbiology. At this point, I have one grant that covers research on central nitrogen metabolism and we study

several aspects of its regulation of nitrogen fixation, one aspect of which was the specific problem that Paul Ludden worked on for his thesis for Burris in the early 1970's.

Nitrogen fixation was something of a backwater topic for a number of years. Indeed, Burris has suggested that he chose the area because it allowed him to do careful science without the complications of a highly competitive environment. However, the combination of the energy crisis of the early 1970's and the advent of cloning methods changed that. For both god and bad political reasons, nitrogen fixation was chosen as a poster topic for the potential of molecular biology and a substantial amount of money became available in the mid-1970's. This drew a large number of chemists, molecular biologists, biochemists and crystallographers to the topic and a great deal of progress was made over the next 20 years. The genes for nitrogen fixation were identified from a number of organisms and the biochemical functions of many were identified. Most critically, Doug Rees at Caltech solved the X-ray crystal structure of dinitrogenase, dinitrogenase reductase and their co-crystal. This also defined the nature of the metal clusters in each of these enzymes. A significant amount of structure function analysis was then performed on these proteins, largely focused on the pathways of electrons through dinitrogenase itself. Finally, the biosynthesis of the metal clusters themselves became intensively studied.

Despite the enormous progress, several things became apparent. First, there was no reason to be optimistic that this basic science was going to fundamentally improve biological nitrogen fixation. That is, there were no obvious ways to improve the catalytic efficiency of the enzyme, nor to substantially improve the amount of fixation, without leading to strain instability. It was also not clear that understanding the nature of the biological catalyst provided good insights in to improvement of chemical catalysts to be used industrially. Indeed, molybdenum-iron catalysts, perhaps by coincidence, had been used in the process for a number of years, though they bore little structural or functional resemblance to the active site of dinitrogenase. Second, the easy (and indeed even the hard) questions had been answered and only the very hard questions remained. For example, the biosynthesis of FeMo-co actually takes place outside of dinitrogenase itself and the finished metal cluster is then inserted into the apo-dinitrogenase by an unknown mechanisms. This biosynthesis involves at least 10 *nif* gene products, and almost certainly non-*nif* proteins as well. Many of these proteins are oxygen-labile as is apo-dinitrogenase itself, so although the general pathway and roles of many of these proteins have been defined, completing the understanding has become technically overwhelming. The field of the biochemistry of nitrogen fixation exists, but it has again become a rather quiet field.

One significant accomplishment of the research outside the specific area of nitrogen fixation was the insight it provided into iron-sulfur cluster assembly at large. It happens that two *nif* gene products, NifS and NifU, are directly involved in Fe-S cluster synthesis and Dennis Dean exploited this to identify the “housekeeping” homologs in *Azotobacter vinelandii* and *E. coli*. Indeed, Tricia Kiley of Biomolecular Chemistry continues to study the role of these proteins in Fe-S cluster biosynthesis in that organism.

Biochemistry of nitrogen fixation

As mentioned above, there is a paradigm system for nitrogen fixation, a few rather subtle

variations on that system and then a completely novel system. We will start with a description of the paradigm Mo-containing system and then treat the others by comparison to that one.

Throughout all this text and indeed in most papers, references to "nitrogenase activity" are rarely to the actual fixation of N_2, though this can be done (typically through the use of $^{13}N_2$). Instead, almost everyone relies on the acetylene reduction assay in which acetylene is reduced by nitrogenase to ethylene (discovered independently by Burris and Dilworth). Both of these are readily separated on a GC (with only a few seconds retention time). This assay can be used in vivo (indeed, both gases readily move through the cell membrane) or in vitro and is linear over at least six orders of magnitude.

The source of reductant:

There certainly must be a source of low potential electrons supplied to dinitrogenase reductase, but definitive proof of that source has only been provided for *Klebsiella pneumoniae*. The "problem" is that virtually any flavodoxin or ferredoxin can serve in this process in vitro. As a consequence, when people fractionated the extracts of a given organism for potential donors to the dinitrogenase system in vitro, a number of distinct protein were identified. Often, one or more of these showed co-regulation with nitrogenase, strongly suggesting a physiological role in the process. However, when mutations eliminating these functions were studied, little or no effect on nitrogenase activity in vivo was detected. In some cases, multiple gene knockouts had a stronger effect on activity. The answer to this mild paradox seems to be that cells have fairly high levels of such low potential electron carriers, at least on conditions conducive to N_2 fixation (i.e. reducing conditions), but they also cause an up-regulation of one or more additional proteins like these to compensate for the potentially high impact on the system of nitrogenase. Thus, when one genetically removes one or even several of these proteins, there is enough activity left to see significant levels of nitrogenase activity in vivo. It simply happens that in *Klebsiella*, the flavodoxin (NifF) plays a larger role in vivo, so that its mutational elimination produces a more striking phenotype.

In *Klebsiella* the source of reductant for Klebsiella NifF is the NifJ protein, a pyruvate:flavodoxin oxidoreductase. This enzyme carries out the oxidative decarboxylation of pyruvate, yielding acetyl-CoA and CO_2.

In fact, almost none of this has been relevant to the in vitro analysis of nitrogenase from any organism as it was recognized early on that dithionite ($S_2O_4^{2-}$) is a highly effective reductant of dinitrogenase directly, as well as serving as a scavenger of dissolved O_2. Other reductants such as sodium borohydride are less effective.

Nitrogenase (dinitrogenase and dinitrogenase reductase)(adapted from J. Nutrition. 2000;130:1081-1084.)

The terminal electron donor to dinitrogenase is a homodimer of roughly 64kDa and is the product of *nifH*. (Fig 2). This protein interacts in an as yet unknown way with low potential electron donors that reduced the Fe-S cluster. The protein then binds two molecules of ATP

The single regular $[Fe_4S_4]$ cubane is symmetrically coordinated between the subunits by Cys97 and Cys132 from each subunit. This $[Fe_4S_4]$ cluster is the redox-active center directly involved with electron transfer to MoFe protein. The $[Fe_4S_4]$ cluster of Fe protein cycles between the reduced (1+) state and the oxidized (2+) state during electron transfer to MoFe protein. An all-ferrous (0) state of Fe protein has also been described, but the physiologic relevance of this

species is unclear. Each Fe protein dimer can bind two nucleotide molecules, at sites distal from the $[Fe_4S_4]$ active site. Binding of MgATP at these sites causes a conformational change in Fe protein. The two subunits rotate toward each other, extruding the $[Fe_4S_4]$ cluster toward the protein surface (and surmised interaction with the P-clusters of MoFe protein) by 4 Å. This conformational change is thought to be a key step in the catalytic cycle of nitrogenase.

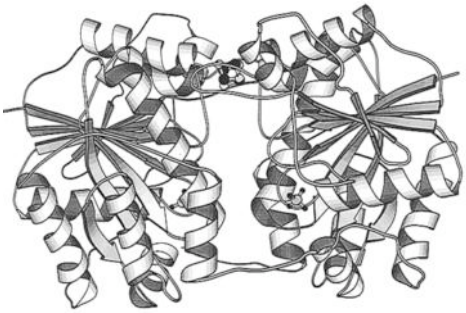
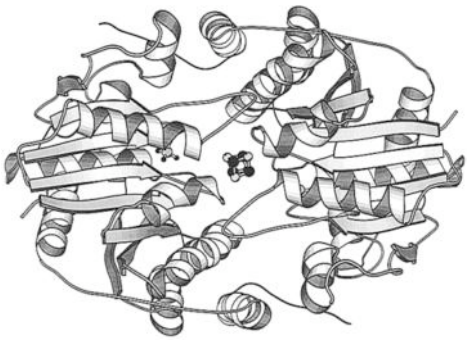


Fig 1 (A, top) Ribbons diagram of the polypeptide fold of the Fe protein dimer from *A. vinelandii,*[30] with ball-and-stick models for the 4Fe-4S cluster and molybdate. The 2-fold axis of the Fe protein dimer is oriented vertically in the plane of the page. (B, bottom) Ribbons diagram of Av2 viewed along the dimer 2-fold axis. Howard and Rees *Chem. Rev.*, **96**, 2965 -2982, 1996



Dinitrogenase (Fig 2)contains the actual site of nitrogen fixation and is a 230-kDa "$_2$\$$_2$ tetramer of the *nifD* and *nifK* gene products. Each MoFe protein tetramer contains two pairs of metalloclusters unique to MoFe protein, i.e., two molydenum-iron-sulfur-homocitrate clusters (FeMo-co)(Fig 3 and 4) and two $[Fe_8S_7]$ clusters (P-cluster) (Fig 3). FeMo-co consists of two partial cubanes ($[Fe_4S_3]$ and $[MoFe_3S_3]$) bridged by three sulfides, with homocitrate coordinated to the Mo atom. FeMo-co is completely encompassed by the three domains of the "subunit and is the presumed site of substrate reduction.

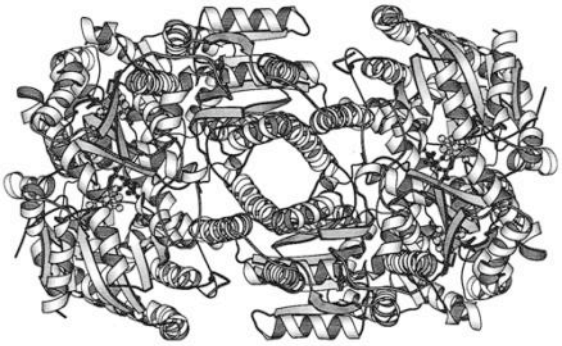
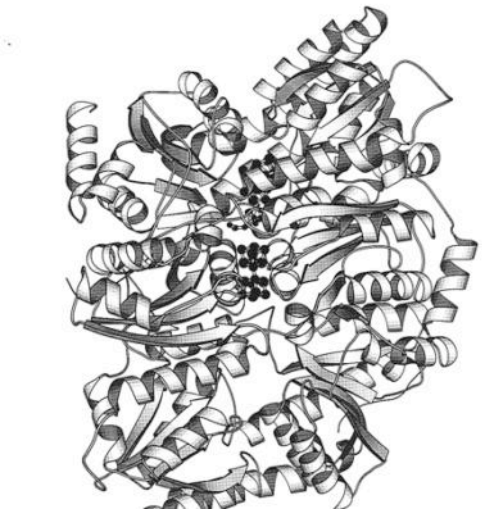


Fig 2. (A, top) Ribbons diagram of the polypeptide fold of the MoFe protein tetramer from *A. vinelandii,*[31,32] with ball-and-stick models for the FeMo cofactors and P clusters. The view is along the tetramer 2-fold axis. (B, bottom) Ribbons diagram of the polypeptide fold of an α\$ subunit pair of the MoFe protein. The view is roughly perpendicular to the view in A, in the direction along the diagonal from the top left to bottom right of the tetramer.



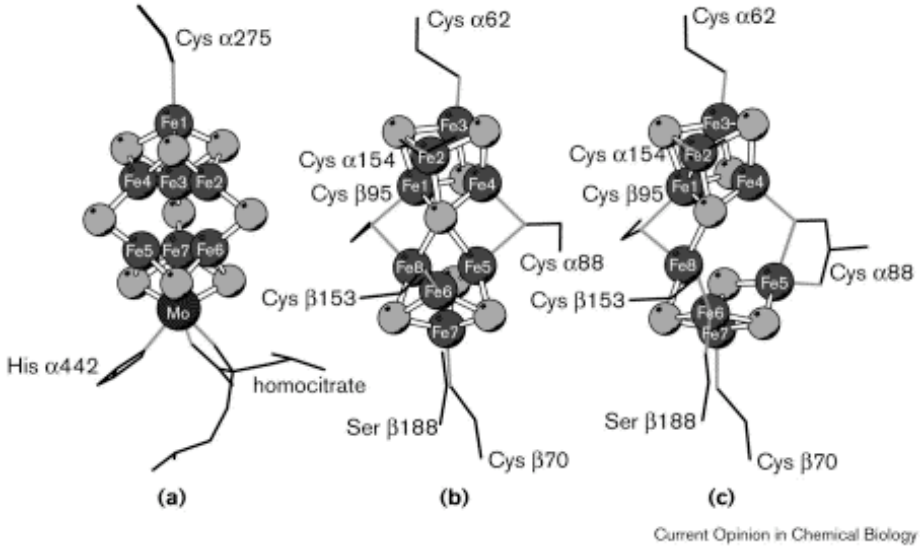


Fig 3. Structural models for the nitrogenase metalloclusters and coordinating ligands. **(a)** FeMo-cofactor. **(b)** The dithionite reduced P^N form of the P-cluster. **(c)** The oxidized P^{OX} state of the P-cluster. Protein Data Bank coordinate sets 3MIN and 2MIN were used for (a), (b), and (c), respectively. from: D. C. Rees and J. B. Howard Curr. Opin. Chem. Biol. 4: 559-566 (2000)

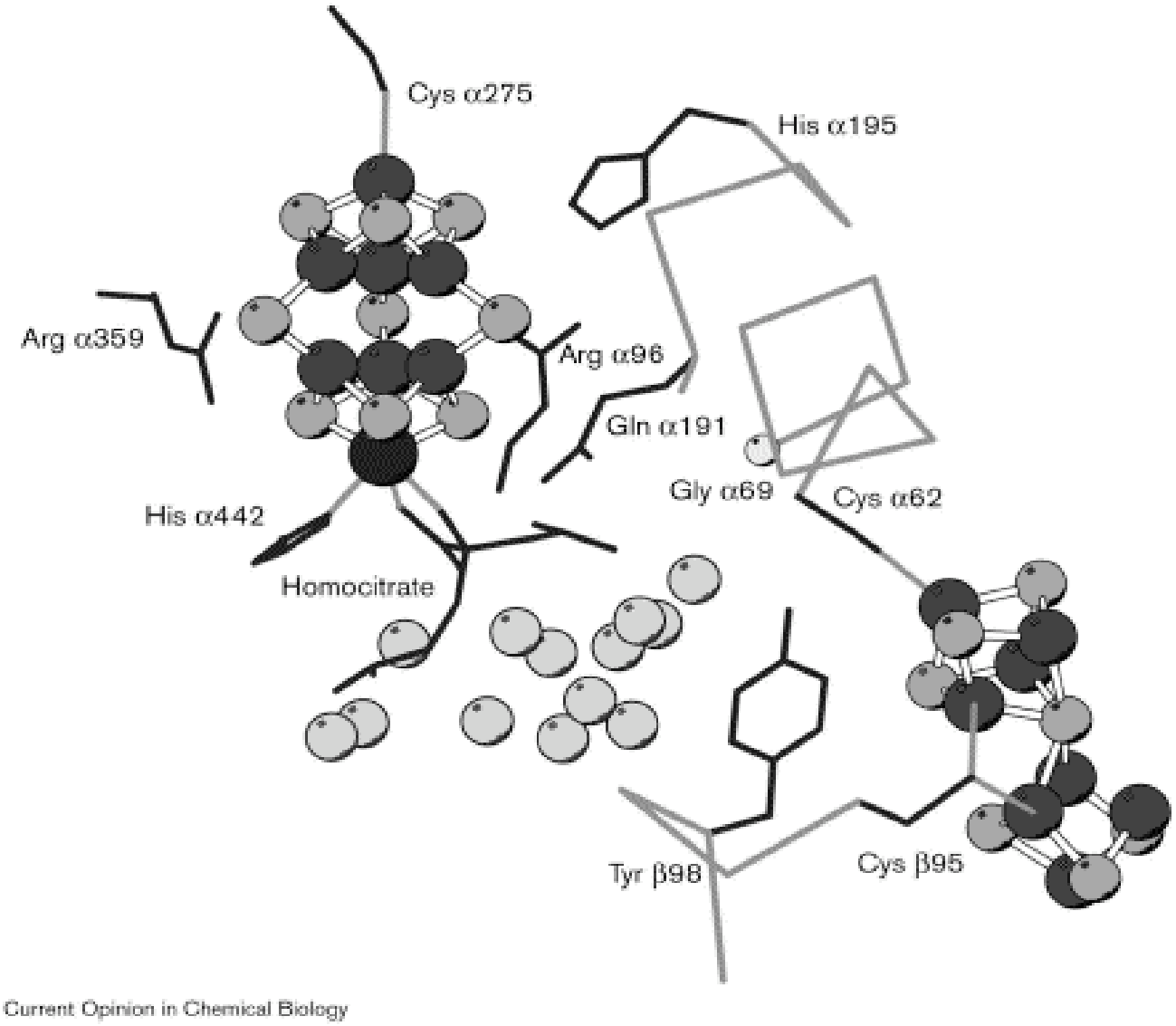


Fig. 4. View of the protein environment between the FeMo-cofactor and P-cluster of the MoFe-protein, including residues that have been studied by mutagenesis. Water molecules near the homocitrate are indicated by isolated spheres. from: D. C. Rees and J. B. Howard Curr. Opin. Chem. Biol. 4: 559-566 (2000)

FeMo-co is not merely a metal cluster of exotic structure, but it also contains an unusual acid - homocitrate. This is actually synthesized by one of the *nif* gene products, NifV, since it is typically absent from most cells. This specific acid is not essential for N_2 fixation, as mutants lacking NifV display a leaky Nif` activity, but that is because one or more other small acids are used in its absence to provide modest function. Rather remarkably, FeMo-co can be purified in an intact, fairly stable form - so long as it is kept strictly anoxic and in an organic solvent like N-methyl formamide. The authenticity of this extracted cluster can be verified not only by distinctive visible and EPR spectra, but by its ability to regenerate the activity of apo-

dinitrogenase (that is dinitrogenase lacking only FeMo-co, isolated from strains lacking one or another of the proteins necessary for FeMo-co synthesis). Now it happens that this is a bit of a fluke because the FeMo-co is near the center of the dinitrogenase structure, so its ability to activate apo-dinitrogenase depends on the presence of an additional protein, discussed below, that apparently holds the apo-dinitrogenase in a structure that allows the addition of FeMo-co. It appears that the normal role of this protein is FeMo-co insertion (discussed below). The precise role of homocitrate in the process remains unclear, because of the site of N_2 binding and therefore catalysis is unknown. (Recognize that the latter problem is probably insoluble: dinitrogenase only binds N_2 when fully reduced, but that means that it immediately begins catalysis upon binding, so the "N_2-bound form" is highly transient.)

Note that the FeMo-co is structurally distinct from the molybdopterin cofactors found in human oxidase enzymes and nitrate reductase in plants. FeMo-co gives rise to the characteristic electron paramagnetic resonance spectrum of MoFe protein. The P-clusters consist of two distorted iron-sulfur partial cubanes with redox-dependent structure. The P-clusters are located at the interface of the " and $ subunits and are intermediates in the electron transport pathway between Fe protein and FeMo-co.

Using data obtained from the nitrogenase systems of *Clostridium pasteurianum*, *Klebsiella pneumoniae* and *Azotobacter vinelandii*, a general sequence of events in the catalytic cycle of nitrogenase can be described. MgATP binding to reduced Fe protein shifts the redox potential of the $[Fe_4S_4]^{2+/1+}$ couple from about -300 mV to nearly -450 mV vs. standard hydrogen electrode. A concomitant MgATP-induced conformational change apparently promotes interaction of Fe protein with MoFe protein. Upon complex formation, an additional conformational change of Fe protein shifts the redox potential of the Fe_4S_4 cluster by an additional -200 mV), making the transfer of a single electron from the Fe protein to MoFe protein energetically favorable. The hydrolysis of MgATP (bound to Fe protein) to MgADP and P_i is coupled to this electron transfer. After electron transfer and MgATP hydrolysis, the nitrogenase complex dissociates in the rate-limiting step of the cycle. Fe protein is then reduced by a low potential electron donor (a ferredoxin or flavodoxin in vivo), and MgADP is exchanged for MgATP. The catalytic cycle is repeated until a sufficient number of electrons have been transferred to completely reduce the FeMo-co-bound substrate. Although Fe protein is the obligate electron donor for MoFe protein in all characterized nitrogenase systems, the in vivo electron donor for Fe protein is less stringently conserved, as noted previously.

Implications of the biochemistry of nitrogenase:

All nitrogenase of this type are O_2-labile, so any cell that wishes to employ the process needs to address that issue. All these nitrogenases are also extremely slow, with turnovers of a few per sec, which means that very large amounts of protein are necessary for growth. This in turn implies that gene regulation is necessary but not sufficient for the regulation of the process - there will simply be too much nitrogenase in the cell under to address by dilution, if a better nitrogen source becomes available. All these enzymes consume substantial amounts of ATP, primarily by the reductase in passing electrons to dinitrogenase. In fact the process is worse than described, because if the flow of reductant is anything but maximal, then the partially reduce dinitrogenase "fixes" two protons with some frequency. In other words, the partially reduced enzyme is not stable and efficient use of reductant requires a fairly rapid achievement of the fully reduced form for N_2 fixation to occur.

There remain many open questions, not the least of which are (i) what is the site of N_2 binding (presumably near the FeMo-co, but…); (ii) how does the conformation change that occurs upon ATP hydrolysis lower the reduction potential of the electrons; and (iii) where are all of the electrons stored on the partially reduced dinitrogenase.

Biosynthesis of dinitrogenase

While we cannot claim to fully understand the biosynthesis of metal clusters, for most of the studied Fe-S clusters, the pattern is simple in that several proteins collaborate to form the metal cluster on the apoenzyme itself. This might well be the process by which the exposed metal cluster of dinitrogenase reductase is formed as well as that of the P clusters of dinitrogenase. However, this is certainly not the situation with the FeMo-co cluster. At least nine separate proteins have been implicated in the process. Perhaps most remarkably, an Fe-only cluster precursor is formed initially on NifB, transferred to NifNE by another protein, where further rearrangement apparently occurs involving dinitrogenase reductase, and then transferred again, possibly to dinitrogenase reductase (?!?!), where Mo and homocitrate become associated. The cluster then appears on an insertase named gamma (Fig 5) . In the presence of apo-dinitrogenase and dinitrogenase reductase, gamma transfers FeMo-co to the apo-enzyme, which has been structurally perturbed to allow access to the center of the protein, then dissociates. The reason behind this remarkably complex scheme is completely unknown, but some other complex heterometal clusters also involve one or more dedicated processing proteins and chaperonins. Note too that dinitrogenase reductase appears at least twice in the process, in addition to its "day job" as terminal reductant for dinitrogenase and a role in regulation as described later.

It remains unclear if the metal centers of either dinitrogenase or dinitrogenase reductase can be recycled when they are damaged. It is interesting to note that the levels of the some of the FeMo-co processing proteins are several per cent of that of nitrogenase itself, consistent with the notion that multiple centers might be synthesized for a given nitrogenase protein.

The alternate nitrogenase that use with Va or Fe in place of Fe appears to have at least some specific processing proteins (Fig 6) that perform analogous functions to those described for the Mo system. You'll note from Fig. 6 that the Va-dinitrogenase (and indeed the Fe-dinitrogenase) actually exist as hexamers, where the major catalytic proteins are homologous to the Mo-dinitrogenase tetramer constituents and the additional subunit (termed _, because of a poor grasp on the Greek alphabet, since _ would have made more sense as the third letter). This additional subunit appear to be analogous to the gamma protein of the Mo-dinitrogenase noted in Fig 5 except that it does not fall off the complex after cluster insertion.

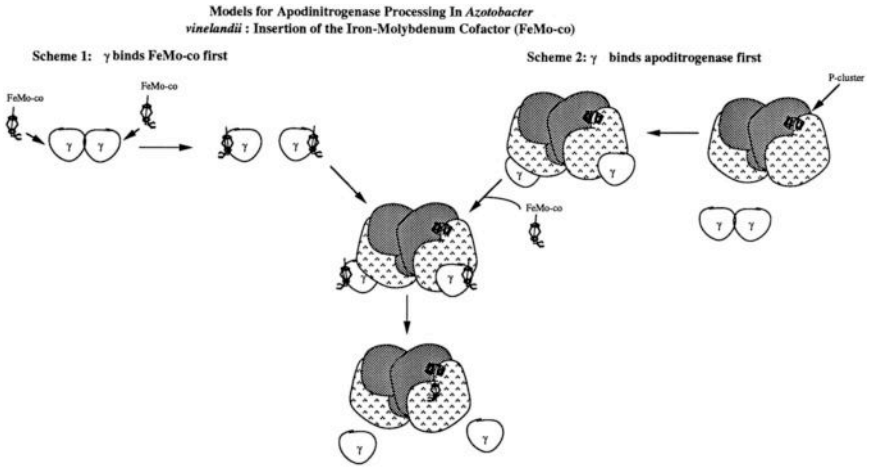


Fig 5: Models for the maturation of dinitrogenase. *Scheme 1* shows the gamma protein binding FeMo-co first and simultaneously monomerizing. With the addition of ATP and dinitrogenase reductase, the gamma-FeMo-co complex can associate with the apodinitrogenase tetramer. The gamma protein then inserts the FeMo-co into apodinitrogenase and subsequently gamma dissociates. *Scheme 2* shows the gamma protein binding to the apodinitrogenase tetramer first, in the presence of ATP and dinitrogenase reductase. FeMo-co then binds to the apodinitrogenase-bound gamma protein. The gamma protein then inserts the FeMo-co into apodinitrogenase and subsequently dissociates. The darker subunits of dinitrogenase represent the β-subunit, while the lighter subunits represent the subunit in which FeMo-co is placed. Only a single FeMo-co and P-cluster for each dinitrogenase tetramer are shown. Homer MJ, Dean DR, Roberts GP. JBC 270: 24745 (1995)

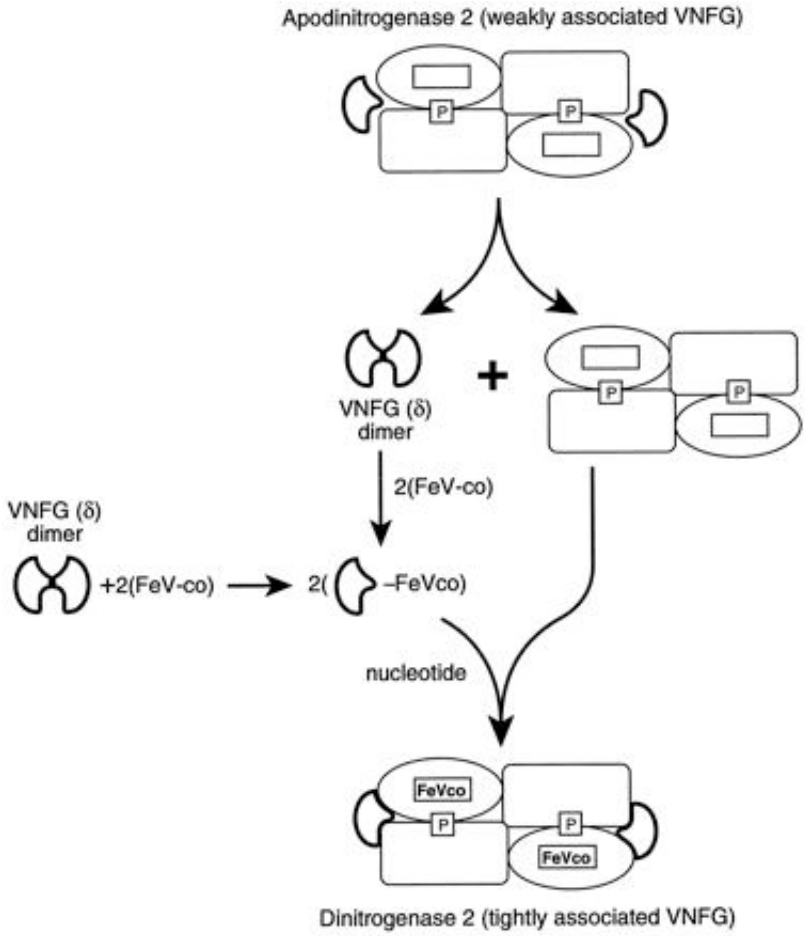


Fig 6. A model for the formation of dinitrogenase 2. The *open squares* represent empty FeV-co sites in the α subunit of apodinitrogenase 2, and the *boxed* "*P*" represent the P clusters. Chatterjee **,** Paul W. Ludden and Vinod K. Shah JBC 272:3758 (1997).

The Meyer nitrogenase:

N_2 Fixation by *Streptomyces thermoautotrophicus* Involves a Molybdenum-Dinitrogenase and a Manganese-Superoxide Oxidoreductase That Couple N_2 Reduction to the Oxidation of Superoxide Produced from O_2 by a Molybdenum-CO Dehydrogenase.N2 Fixation by Streptomyces thermoautotrophicus Involves a Molybdenum-Dinitrogenase and a Manganese-Superoxide Oxidoreductase That Couple N2 Reduction to the Oxidation of Superoxide Produced from O2 by a Molybdenum-CO Dehydrogenase* Ribbe, Gadkari and Meyer. J. Biol. Chem. 272: 26627-26633 (1997).

Streptomyces thermoautotrophicus UBT1 occurs in natural enrichments in the covering soil of burning charcoal piles. The bacterium is characterized by the utilization of gases as sources of energy, carbon and nitrogen (gasotrophy). *S. thermoautotrophicus* grows with CO or H_2 plus CO_2 under aerobic, chemolithoautotrophic, and thermophilic conditions. It is a free living dinitrogen fixer. Although CO and H_2 are known as inhibitors of nitrogenase activity,

S. thermoautotrophicus is able to fix N_2 with CO or H_2 plus CO_2 as growth substrates as well as in the presence of CO plus ethine. The N_2-fixing system of *S. thermoautotrophicus* is expressed constitutively. Intact bacteria reduced ethine to ethene at a negligible level of less than 0.001% of the activity of *Azotobacter vinelandii*.

Table I Contents of metals, acid-labile sulfide, and sulfhydryls of St1 and St2 protein from S. thermoautotrophicus

Protein	Metals					Acid-labile sulfide	Sulfhydryls	
	Mo	Fe	Zn	Mn	V		Available	Total
	mol/mol protein							
St1	0.7 ± 0.1	12 ± 1	3.4	ND[c]	<0.06	8 ± 1	4 ± 0.1	12 ± 1
St2	<0.04	<0.07	0.1	0.24 ± 0.03	<0.02	<0.02	0	0

Molecular masses of 144 kDa (St1) and 48 kDa (St2) have been used for calculations. The values of acid-labile sulfide, molybdenum, and iron are the mean of six, manganese the mean of two and the sulfhydryls the mean of four independent determinations.

[c] Not determined.

In assays with limiting amounts of N_2 in helium, 1 mol of N_2 was reduced yielding 2.2 ± 0.2 mol of NH_4^+ and 0.9 ± 0.2 mol of H_2. The data would support the following equation for the reduction of N_2 by the proteins St1 and St2

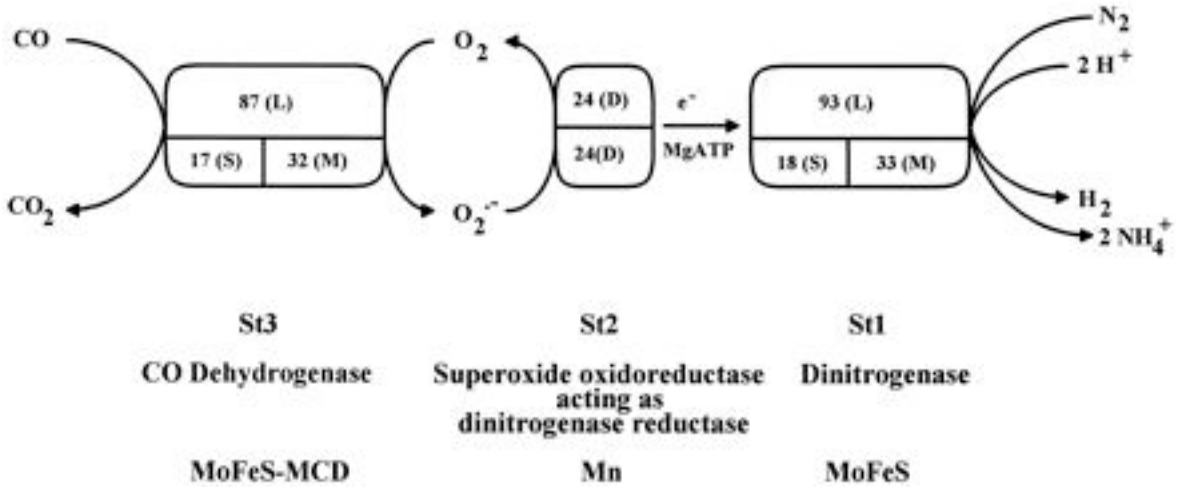


Fig 7. Schematic representation of N_2 fixation in *S. thermoautotrophicus*. Superoxide is produced by CO dehydrogenase through the oxidation of CO and the transfer of the electrons to O_2. Subsequently, the superoxide is reoxidized by a superoxide oxidoreductase that delivers the electrons to a dinitrogenase. The dinitrogenase is capable of reducing N_2 and H^+, but not ethine. The numbers refer to the *MW*s of subunits in kDa. *MCD*, molybdopterin cytosine dinucleotide. The stoichiometries given in the scheme are arbitrary.

Table II. Nitrogenase activities in cytoplasmic fractions of *S. thermoautotrophicus* under different assay conditions

Conditions	Nitrogenase activity
CO plus air	22.6
CO plus air plus SOD (*E. coli* or *B. stearothermophilus*)	0.0
H_2 plus air	25.2
Xanthine oxidase plus xanthine plus air	35.3
Xanthine oxidase plus hypoxanthine plus air	32.1
Riboflavin plus light plus air	20.6
CO plus N_2	0.0
CO plus N_2, O_2 was added after 3 h	33.2

DISCUSSION N_2 fixation by *S. thermoautotrophicus* involves CO dehydrogenase, free O_2, and $O_2^{-\cdot}$ (superoxide) as electron carriers and the proteins St2 and St1 (Fig. 7). CO dehydrogenase generates superoxide anion radicals ($O_2^{-\cdot}$) from O_2, which makes N_2 fixation in *S. thermoautotrophicus* obligately O_2-dependent and establishes a molecular coupling via $O_2^{-\cdot}$, which is in contrast to the electronic coupling in the known nitrogenase systems. The $O_2^{-\cdot}$ anions

are free intermediates and can be trapped by SOD (Table II). Consequently, interaction of CO dehydrogenase and the St2 protein for $O_2^{-\cdot}$ transfer is not required. Generally, $O_2^{-\cdot}$ is considered a highly reactive and destructive metabolic by-product that requires detoxification, *e.g.* by superoxide dismutases. N_2 fixation in *S. thermoautotrophicus* shows that the redox couple $O_2/ O_2^{-\cdot}$ ($E_{0\prime}0 = -0160$ mV) operates as an electron carrier with similar efficiency as ferredoxins, flavodoxins, or hydroquinones in the known nitrogenase systems. In addition, the use of $O_2^{-\cdot}$ in N_2 fixation by *S. thermoautotrophicus* seems to be a powerful mechanism to scavenge $O_2^{-\cdot}$ radicals.

The dinitrogenase reductases known so far are _2 dimeric iron proteins with molecular mass ~63 kDa and contain 4Fe and 4S atoms per dimer. In contrast, the St2 protein of *S. thermoautotrophicus* has been identified as a manganese-superoxide oxidoreductase with molecular mass ~48 kDa and does not contain Fe or S (Table I). In contrast to SOD, St2 is unable to disproportionate $O_2^{-\cdot}$ into O_2 and H_2O_2. The function of St2 is rather to generate electrons through the reoxidation of $O_2^{-\cdot}$ ions to O_2 and to deliver them to the St1 protein (Fig. 7). Like the known nitrogenases, the *S. thermoautotrophicus* system also has a requirement for MgATP. With *S. thermoautotrophicus* nitrogenase the most efficient MgATP/N_2 ratio is 4, instead of 16 reported for nitrogenases from other sources, indicating its superb efficiency. Future work must unravel how MgATP interacts with the nitrogenase proteins.

It is likely that the reduction of N_2 takes place at the St1 protein of *S. thermoautotrophicus*. The St1 protein was also identified as a MoFeS-protein but with molecular mass ~144 kDa and a differing LMS heterotrimeric subunit structure. It revealed per molybdenum atom, 13.8-21.7 iron atoms and 8.8-15 acid-labile sulfide atoms (Table I). There was a moderate sequence similarity between the N-terminal sequences of the subunits St1-L and Kp1-_ or St1-M and Kp1-α, although we are aware of the limitations of a comparison of subunit N termini. The overall reaction catalyzed by *S. thermoautotrophicus* nitrogenase compares to that of known nitrogenases, including the concomitant formation of H_2.

Regulation

The *Klebsiella* paradigm

The paradigm for regulation is the nif system of *Klebsiella pneumoniae*, which is a close relative of *E. coli* and *Salmonella enterica* (Fig 8). This organism does seem to engage in some associative nitrogen fixation with certain grasses, but it is hardly of major agricultural significance. Rather, it was its similarity to *E. coli*, which allowed the use of a number of standard genetic methods, that made it the organism of choice to study regulation and the genetics of nitrogen fixation.

Klebsiella has 26 genes in a single cluster of 7 operons. Because of the extreme expense of synthesizing and running the nitrogenase system, the genes are not expressed unless there is extreme nitrogen deprivation. Under such conditions, the global nitrogen regulatory system causes the autophosphorylation of NtrB, which in turn phosphorylates NtrC, a transcriptional activator. This protein interacts with RNAP bearing the unusual F^{54} sigma factor and then stimulates the expression of the *nifLA* operon in the nif cluster. It appears that expression of *nifLA* occurs only after a prolonged time of nitrogen starvation, presumably to allow the cell to

test for the presence of any other conceivable nitrogen source, but the mechanism of the delay in expression is unknown. NifL and NifA are themselves a two-component regulatory system, albeit a highly unusual one: NifL is the sensor, but is not membrane associated, nor does it autophosphorylate itself or NifA. Instead, NifL binds to and inhibits NifA under appropriate conditions and NifA in the absence of NifL is active. (The divergence of NifLA from the two-component paradigm is especially amusing because the notion of two-component system was first proposed after a comparison of NIfLA and NtrBC, albeit on rather flawed experimental grounds.)

It appears that the issue of nitrogen starvation is determined solely by the global system, and the role of NifLA is two-fold. First NifL is an O_2-sensor and bears a redox-sensitive flavin. Under reducing conditions it forms a fairly stable complex with NifA, preventing the function of that activator. N-regulation is effected by an interaction with GlnK, part of the global nitrogen regulation system (Fig. 9). Separately, NifA is itself temperature-sensitive and has little activity above 32 ^{o}C, though the nitrogenase system of *K. pneumoniae* appears to function at least to the upper 30^{o} s, so the physiological reason for this regulation is unclear (the generally similar system of *Azotobacter vinelandii* is shown in Fig. 9).

If conditions are appropriate, such that NifL fails to interfere with NifA, then NifA also interacts with RNAP-F^{54} and activates expression from the promoters of the other six *nif* operons. Because of the slow turnover of nitrogenase, the levels of nitrogenase protein required for even minimal growth are substantial - several percent of total cell protein. Additionally, a substantial amount of the auxiliary proteins for maturation of nitrogenase and for electron transport are also necessary.

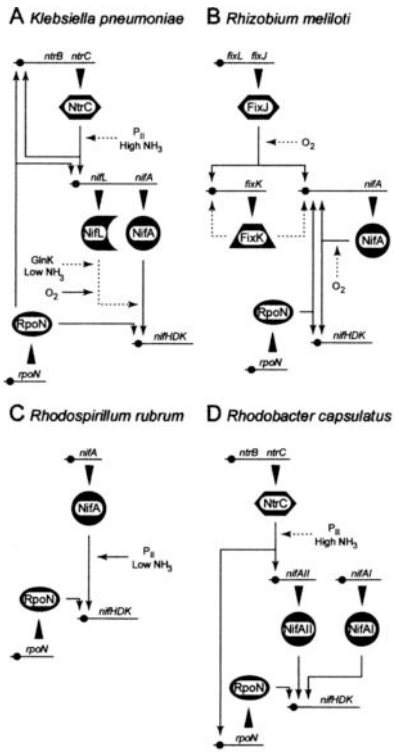


Fig. 8. Comparative models of transcriptional regulation of *nif* genes in (*A*) *Klebsiella pneumoniae*, (*B*) *Rhizobium meliloti*, (*C*) *Rhodospirillum rubrum* and (*D*) *Rhodobacter capsulatus*. PII (the *glnB* gene product) and GlnK are global nitrogen regulation proteins of similar structure. RpoN is the _^{54} subunit of the RNA

polymerase complex. Functionally similar regulatory proteins are symbolized identically in each panel. Solid and dashed lines indicate positive and negative regulation, respectively. Solid circles indicate promoter sites.

For unexplained reasons, the *nif* mRNAs are surprisingly stable (20 min half-lives) under nitrogen-fixing conditions in *K. pneumoniae* and apparently in *Azotobacter vinelandii* as well. This is in contrast to the 2-3 minute half-lives of most bacterial mRNAs, though the raw numbers are perhaps a bit deceptive and it might be good to recognize that cell generation times are long under these conditions as well, so as a fraction of a generation time, the mRNAs might not be so impressively stable.

So what happens to *Klebsiella* if it is fixing nitrogen and a better nitrogen source appears (or the environment becomes aerobic)? Certainly the transcriptional regulation noted above shuts gene expression, but there are still the stable mRNAs producing protein. Surprisingly, these mRNAs become rapidly destabilized, falling to a half-life of about 2 minutes and this destabilization requires the presence of NifL, though the mechanism is not known. There is an additional, and very significant problem, however, the cell still has massive amount of nitrogenase that is catalytically active, but no longer necessary. Transcriptional or post-transcriptional regulation do not address this issue. Instead, there is a fairly substantially drop in the flow of reductant to nitrogenase - again by an unclear mechanism - which therefore stops the hydrolysis of ATP and the catalytic turnover. Somewhat surprisingly, the proteins themselves are not rapidly turned over, but appear to disappear by general dilution.

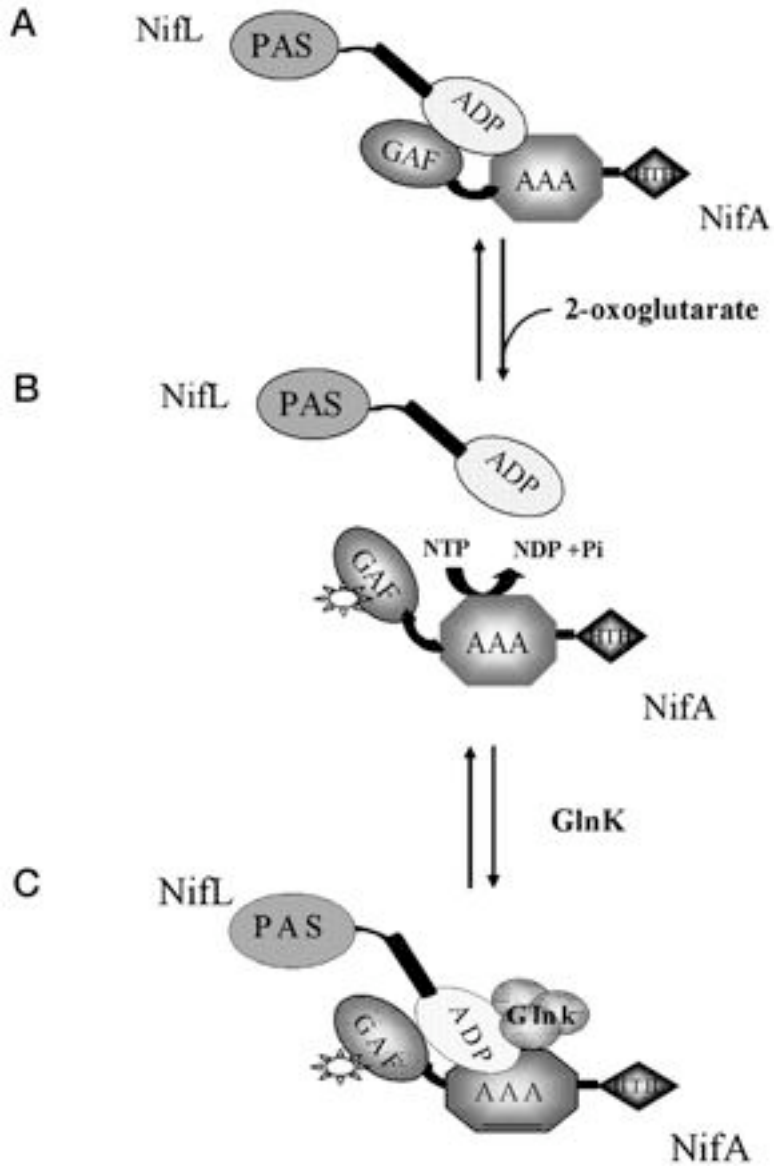


Fig. 9 Model for regulation of NifA activity in response to NifL, Av GlnK, and 2-oxoglutarate. The PAS and ADP-binding domains of NifL are shown as *gray* and *light gray ovals*, respectively. The GAF and AAA+ domains of NifA are depicted in *dark gray*, and the DNA-binding domain with the HTH motif is shown as a *diamond. A*, in the presence of ADP, NifL binds to NifA, inhibiting its activity. *B*, upon binding of 2-oxoglutarate (depicted as a *star*) to the GAF domain of NifA, NifL is no longer competent to inhibit NifA activity. *C*, the interaction of the non-modified form of Av GlnK with the carboxyl-terminal domain of NifL restores inhibition even when the 2-oxoglutarate concentration is saturating. Little and Dixon J. Biol. Chem., 278: 28711-28718 (2003)

Regulation: Other patterns

Oxygen: In general, the decision to express the *nif* genes is highly dependent on the absence of other fixed nitrogen sources in all studied organisms. Though the precise mechanisms for this regulation differs somewhat, the involvement of elements of central nitrogen regulation is common and unsurprising. Oxygen regulation is rather different, in part because different

diazotrophs see oxygen in very different ways. Some diazotrophs are obligate anaerobes, so the issue of oxygen simply never comes up, at least not in a *nif*-specific sense. Rather amazingly, some diazotrophs are obligate aerobes, so they too ignore oxygen as a regulatory signal (discussed below). At least some cyanobacteria, such as *Synechococcus*, solve the dilemma of performing nitrogen fixation in the same cell as O_2 producing photosynthesis by separating the two processes in time. These organisms set up a circadian rhythm by which they only fix N_2 at night when oxygenic photosynthesis is not taking place. A completely different scheme must be in place with *Trichodesmium*, mentioned above for the role in N_2 fixation in oceans. These cells also show a circadian rhythm, but one in which nitrogen fixation occurs concurrently with photosynthesis, but the basis of the protection of the nitrogenase from O_2 is unknown: it might be partly structural or metabolic (such as extreme respiration rates) or a combination of factors.

As described below, still other organisms create environments for their nitrogenases that protect them from oxygen (indeed, this is what the aerobes do as well, though by a somewhat different route), so the creation of these structures is regulated even if nitrogenase is not in a formal sense.

Posttranscriptional regulation: While mRNA stability and its regulation have simply not been examined in most diazotrophs, there is no question that posttranslational regulation of nitrogenase activity is a very common regulatory scheme. The obvious reason is that it is rapid and almost any organism might find itself in an environment where massive amounts of nitrogenase activity no longer make sense. In most cases, the mechanism involves stoppage of the flow of electrons to nitrogenase, but the details can be very different. One of the best understood systems of post-translational regulation exists in *Rhodospirillum rubrum*, which is a gram-negative phototroph (Fig 10). It decides to lower the level of nitrogen fixation in response to two environmental conditions: the appearance of fixed nitrogen or the loss of energy, which in this case refers to the removal of light.

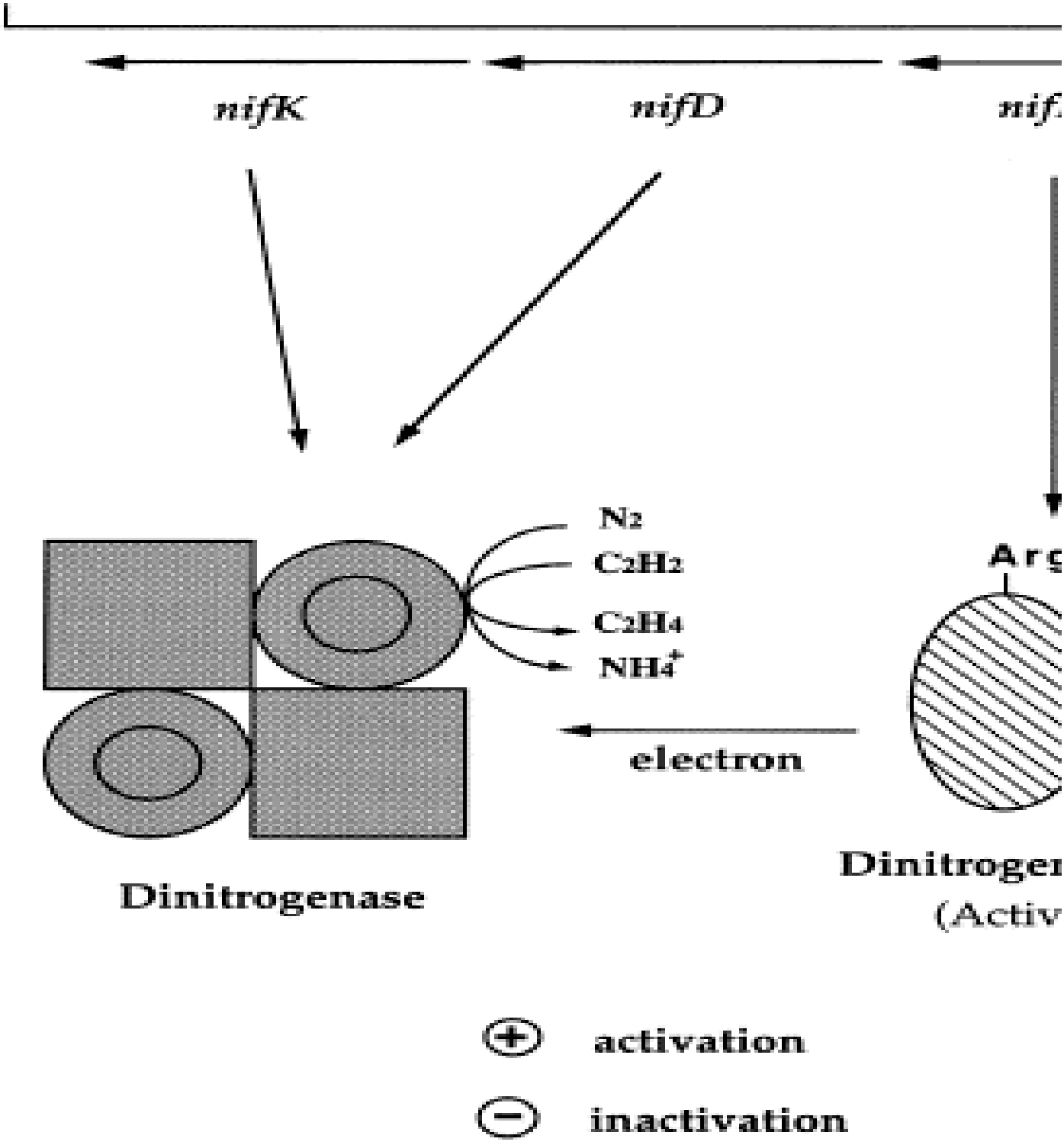


Fig. 10. Model for the regulation of nitrogenase activity by ADP-ribosylation of dinitrogenase reductase. In *A. brasilense*, ADP-ribosylation of dinitrogenase reductase is catalyzed by the DRAT/DRAG regulatory system. In response to exogenous fixed nitrogen or shifting cells from microaerobic to anaerobic

conditions, DRAT catalyzes the transfer of ADP-ribose from NAD to the Arg-101 residue of one subunit of the dinitrogenase reductase, thus inactivating that enzyme. When NH_4^+ is exhausted or cells are returned to microaerobic conditions, DRAG removes the ADP-ribose from the covalently modified dinitrogenase reductase, and restores its activity. Zhang, Burris, Ludden and Roberts FEMS Micro Lett 152:195 [1997]. Both DRAG and DRAT are themselves post-translationally regulated. partly by PII homologs that sense N status, and partly be the redox state of their substrate, dinitrogenase reductase.

Metals:

The Mo dinitrogenase is the most efficient and, not surprisingly, in those microbes with more than one set of genes for different nitrogenases, the genes for the Mo system will be turned on preferentially. However, the molecular basis for this regulation has proven to be very difficult to tease out. In some organisms like *Rhodospirillum rubrum*, it appears that the activity of the Mo system is sensed in some way, since the Fe nitrogenase in that organism is synthesized with the media lacks Mo or when a mutation destroys the activity of the Mo enzyme. In other bacteria, there is a specific metal-sensing system such that the presence of Mo blocks the expression of the alternate nitrogenase systems irrespective of the functionality of the Mo nitrogenase. The genes for the alternate system tend to have a dedicated NifA-like positive regulator and there is some evidence for a role of dinitrogenase reductase (or other of the homologs) in the expression of the alternate systems (as if this protein needed other activities).

Structural solutions to the oxygen problem:

Azotobacter vinelandii is an obligate aerobe that performs nitrogen fixation whenever fixed nitrogen is limiting. It solves the O_2 problem by at least two mechanisms. First, it maintains exceedingly high rates of respiration, so that the levels of dissolved O_2 in the cytoplasm are apparently quite low. Secondly, it has several specific proteins that associate with nitrogenase in such a way to physically protect it from O_2 damage. In crude extracts of *Azotobacter*, nitrogenase therefore displays only modest tolerance for O_2, but upon purification (away from the protecting proteins) nitrogenase of this organism shows typical O_2 sensitivity.

A much more elaborate solution is that of some of the filamentous cyanobacteria. These organisms form long chains of cells that, under conditions of nitrogen sufficiency, are all vegetative growing cells. Upon nitrogen starvation, however, cells at periodic positions along the filament (with the frequency depending on the specific species), certain cells undergo a profound metamorphosis. They become impermeable to O_2, or anything else, except through the contacts with the cells on either side in the chain. These cells are termed heterocysts and are terminally differentiated - they are not able to further divide. Instead they accumulate high levels of nitrogenase and process N_2 obtained from the neighboring ells and convert that into NH_3, which then serves to support the growth for several cells on either side. The vegetative cells continue to grow and when the distance between two heterocysts becomes too large to efficiently provide fixed nitrogen to all the cells in between, a cell in the middle then differentiates.

Frankia is a diazotrophic actinomycete that forms vesicles along its filamentous structure that have a very different form, but roughly the same role, as do the heterocysts of the cyanobacteria,

Symbiosis

Symbioses between bacteria and plants are numerous and complex, but the central issue is always the same: when nitrogen limited, plants encourage the infection of a very specific bacterial type and sequesters these bacteria inside a specialized structure called the nodule. It then supplies carbon and energy to the bacteria which in turn produce fixed nitrogen for the plant. Presumably the advantage to the bacterium is that at some point the nodules fall apart and allow the bacteria to enrich themselves in their environment. The bacteria can infect plants either through the roots or the stem and such bacteria are currently classified in to 27 species in 6 genera. There have been a number of name changes for these organisms, particularly as molecular phylogeny has been applied.

The proper symbiotic pair is set up through a remarkable dialog between bacteria and plant (Fig. 11). Different plants excrete specific flavonoids or related compounds, which are sensed by the appropriate bacteria. These “nodulation gene inducers” have a variety of heterocyclic structures, though a few are non-flavonoids. In response to binding these flavonoids to specific receptors (typically referred to as the NodD proteins), the bacteria then express proteins that produce the bacterial side of the conversation, the Nod factors. Nod factors are lipo-oligosaccharides 4-5 units long with a backbone of $-1-4 linked N-acetylglucosamine units that are appropriately decorated with modifications and then secreted. A number of bacterial genes are necessary for their biosynthesis.

The secreted Nod factors are then recognized by the plant at very low concentrations, though the nature of the receptor proteins is still being worked out. Indeed, it appears probably that more than one different receptor might be involved and there is some confusion over how specific at least some of the interactions might be. It is safe to say, however, that it is not a simple one-to-one recognition.

Here is a general description of the process, though the specific details vary with different symbioses: The response of the plant to the presence of the bacteria is rapid, starting with Ca^{2+} influx and C1 efflux from the root hair cells, where the bacteria attach. Within 10 minutes, the root hairs deform, creating a “shepherd’s crook” that facilitates bacterial infection. Within 24 h, there is a visible “infection thread which is a bacteria-filled tube running from the site of attachment to a particular plant cell, where division has begun. They are they released from the thread endocytotically from "droplets" at the unwalled tips of the threads. This results in the bacteria existing with a "peribacteroid" membrane within the nodule cell cytosol, where they undergo at least one round of cell division. They then undergo a remarkable transformation in to what are termed bacteroids, which is a terminally differentiated state that is the form in which they fix N_2. Some of these peribacteroid membranes fuse to form larger assemblages of bacteroids. Within 4 days, small visible nodules are formed containing bacteria. These nodules vary a bit among different plant species. In some species there is a clear organizational pattern of differentiation across the nodule, with vegetative cells at one end, developing bacteroids and functional bacteroids in the middle and senescent bacteroids at the far end, but in others the overall organization is less distinct.

It appears that relatively few bacteria are actually taken up at the site of attachment, as revealed by the fact that infection of plants with mixed population of bacteria often results in a single species in a single nodule. For this and other reasons, there must necessarily be a competition for attachment among bacteria and the more competitive bacteria are not necessarily the most effective (in terms of nitrogen fixation). At least some rhizobia have been shown to

perform quorum-sensing, which is presumably involved in competitiveness.

One of the more amazing features of the nodule is the presence of leghemoglobin, an O_2-transporter produced primarily in infected plant cells. The role of this protein is to bind O_2 with high affinity and the cell surface and then transport that O_2 to the bacteroids. This allows aerobic respiration without the problem of free O_2 damaging nitrogenase. The source of carbon and energy provided by the plant is typically dicarboxylic acids, such as malate.

Nitrogen fixation produces NH_3, but the metabolite that is provided to the plant is apparently alanine in soybeans, and ureides, allantoin and allantoic acid in many tropical legumes. In this case, the NH_3 is processed through glutamine synthetase and glutamate synthase to glutamine, which is further processed to glycine and aspartate. These then feed into a purine biosynthesis pathway to yield uric acid which is oxidized to allantoin.

Actinorhizal plant symbioses:

Frankia are diazotrophic actinomycetes that form root nodules on eight families of angiosperms, though the specific set of infected plants are defined largely by their ability to be infected. The two best known are alder (*Alnus*) and laurel (*Myrica*). There has clearly been co-evolution between the plants and the bacteria, but the relationships are complicated and the slow growth of the bacteria has caused them to be under-studied.

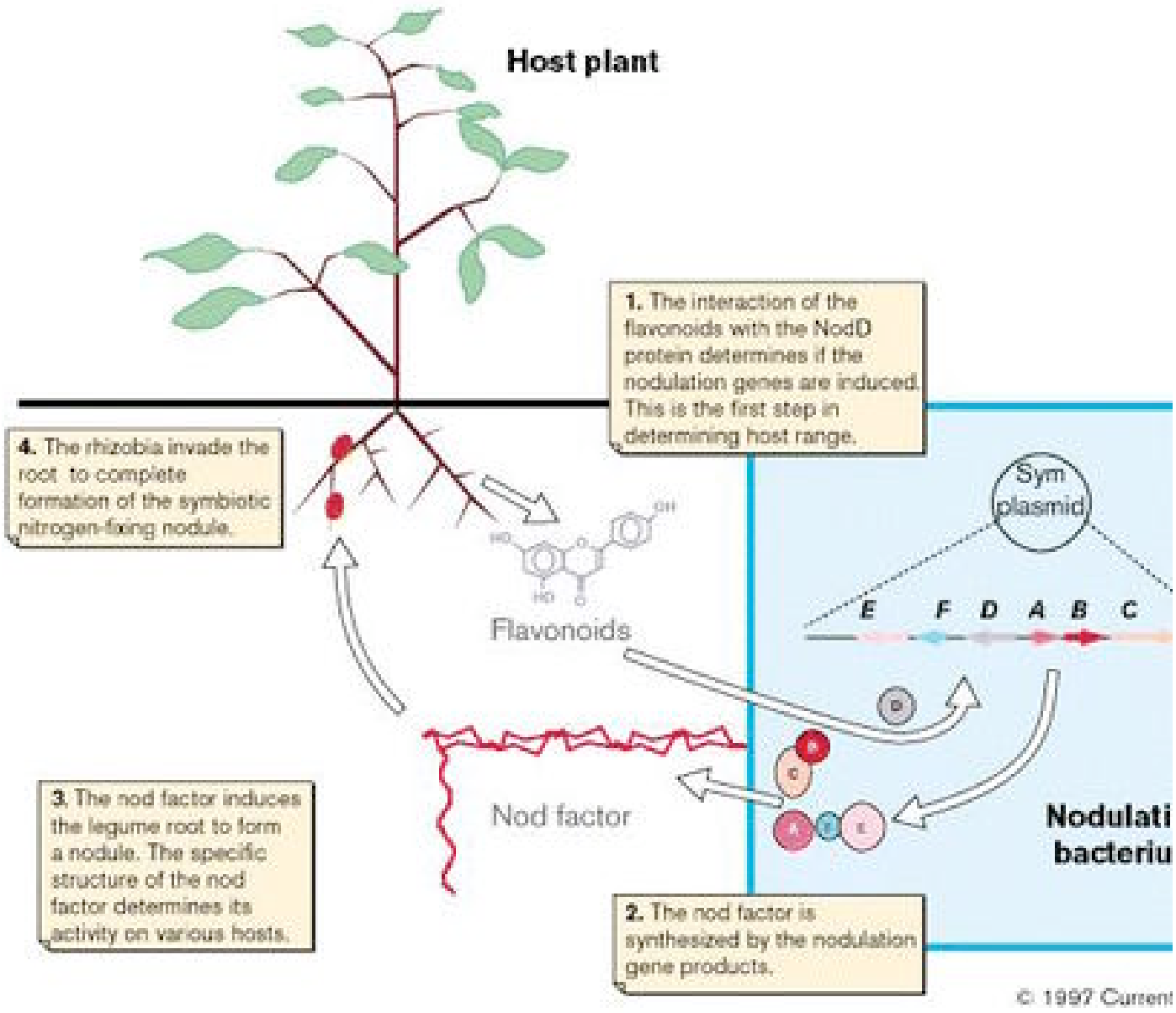


Fig 11. The major steps in formation of nitrogen-fixing nodules in *Rhizobium*–legume symbiosis. As shown, the nodulation genes of the bacterium are linked and carried on a 'Sym' plasmid.

Associative nitrogen fixation:

There are other plant-bacterial interactions that are less specific and certainly less biochemically involved than the ones discussed above. These come under the general heading of "associative" since the bacteria are rather loosely associate with the plant. Nevertheless, these are almost certainly of ecological importance. They include the association of *Azospirillum*, *Acinetobacter, Herbaspirillum* and *Azoarcus* with sugar cane, kallar grass and rice, as well as

with other grasses and cereals. In some cases, substantial populations of bacteria can be found living within the plant tissue having arrived there through cracks, but in other cases there is simply a close physical contact with the roots. In many of these cases, a direct role of nitrogen fixation has been established, but it seems likely that the bacteria often provide other growth stimulants as well, including the provision of plant auxins.

Current interest and value

As noted above, the research has been quite useful in a variety of areas of basic biology, including infection processes, chemical catalysis and metal processing. However, there has not been, nor is there reason to expect that there will be, an important new symbiosis of agricultural created from our knowledge, nor the dramatic improvement of an existing one. Either of these might of course happen, but the research has demonstrated how very complex each of these processes are and therefore how unlikely it will be for someone to create a new one.

A more likely avenue of actual application, as an guess, will be to better understand associative nitrogen-fixing situations and try to improve them. These have the advantage that the requirements for the interaction are rather lower than the nodule-formation processes, which means that new interaction of this sort might be feasible, or at least the microbe in question might be made into a more helpful collaborator of the plant. Even this goal might, however, not be in the cards. It is also unlikely that our understanding of the biological process will lead to a better industrial catalyst. After all, biology uses protein structure, even in the context of catalytic metal clusters, for a reason.

Interestingly, one of the major outcomes of the research has not been on the applications which were the political driving force, but (in the finest tradition of basic science) as a good model system for metal processing and metal cluster assembly. This is because the enzyme is slow relative to its required function that fairly massive amounts are required, which means that proportionately massive amounts of processing enzymes are also required. This is turn has made biochemical analysis a bit easier than it would otherwise be. Also, because nitrogen fixation was not essential for cell survival (unlike some central metal processing systems might be) we have been able to exploit classical genetic phenotype in unraveling the system.