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BIOLOGICAL NITROGEN FIXATION

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INTRODUCTION

Nitrogen is a key macro element required by all living organisms. However, a broad spectrum of nitrogenous compounds are usable by different organisms. Higher plants and bacteria can utilize diverse organic and inorganic nitrogenous compounds, and some prokaryotic organisms can use N_2 via biological N_2 fixation. Animals are the most demanding organisms and require eight to ten preformed amino acids for their nutrition. They discard nitrogenous metabolic products that serve as excellent sources of nitrogen for the growth of microorganisms and plants. Not only are the animals of the planet directly dependent upon the photosynthetic activity of plants for their energy, but they also are dependent directly or indirectly upon plants for many of their nitrogenous compounds as well. The higher plants in turn depend upon nitrogenous compounds derived from N_2 by chemical fixation (primarily through the Haber-Bosch process) or by biological N_2 fixation.

CHEMICAL FIXATION OF N_2

Chemical fixation of N_2 requires the use of fossil fuels as a source of H_2 and of energy. Natural gas commonly provides both H_2 as reductant and, by combustion, the high temperature required for the catalytic production of NH_3 from N_2 at high pressure. The supply of natural gas is finite, and coal probably will be substituted as the feed stock for chemical N_2 fixation when natural gas is exhausted.

BIOLOGICAL FIXATION OF N_2

Biological N_2 fixation, like chemical fixation, is energy demanding, but solar energy can be tapped by green plants and some bacteria to fuel fixation. Thus any increase in exploitation of biological N_2 fixation can help to slow the depletion of our fossil fuels. Chemical fixation of N_2 yields about 60×10^6 metric tons of fixed nitrogen per year (29), but this major chemical industry contributes much less fixed nitrogen to the earth's nitrogen cycle than does biological fixation. Estimation of annual biological fixation of N_2 is very difficult, and hence estimates vary widely—from 100×10^6 to 180×10^6 metric tons per year (8, 16, 47). Although disagreement on the magnitude of biological N_2 fixation is widespread, there is agreement that it is two or more times greater than chemical fixation.

If we examine the nitrogen cycle on earth, we recognize that the atmosphere constitutes an enormous reservoir of N_2. It can be chemically or biologically reduced so that it can function in living organisms. It can be discharged as

soluble nitrogenous compounds that are lost from the terrestrial reservoir to the sea or it can be bound to particles, primarily in the soil. Denitrification can return N_2 to the atmosphere from the terrestrial cycle.

Agents

Centuries ago farmers recognized empirically that it was beneficial to mix N_2-fixing crops with crops incapable of fixing N_2, although the basis of the enhanced growth was not apparent. In the 1830s Boussingault (67) reported that leguminous plants fix N_2. This was contested by Liebig (67) in Germany, and Lawes, Gilbert & Pugh (67) in Great Britain. Experiments of Ville (67) in France and tests elsewhere did not resolve the problem to everyone's satisfaction. Finally, Hellriegel & Wilfarth reported in 1886 and published in 1888 (67) convincing evidence for N_2 fixation in leguminous plants, and they localized fixation in the root nodules of pea plants. Their findings sparked interest in the agronomic application of inoculation for legumes and in studies of the process of nodulation and the structure of root nodules. These nodules contain large numbers of the rhizobia in a modified form referred to as bacteroids. They receive abundant photosynthate from the plant to supply their energy requirements for N_2 fixation.

Somewhat later, biological N_2 fixation was demonstrated in free-living microorganisms: anaerobic fixation in *Clostridium pasteurianum* (68), and aerobic fixation in *Azotobacter chroococcum* (4). Since then a variety of other prokaryotic organisms (but no eukaryotes) have been added to the list of N_2 fixers. These include cyanobacteria, photosynthetic bacteria, *Klebsiella* spp., archaebacteria, *Desulfovibrio* spp., etc. (70). Symbiotic systems in which nodules are formed and fix N_2 on the root systems of nonleguminous plants (e.g. on the roots of alders by *Frankia* spp.) are widespread (53). In recent years considerable interest has focused on associative fixation between a nonsymbiotic microorganism (e.g. *Azospirillum* spp.) growing on the root system of a nonleguminous plant but without forming nodules (19). Young (70) has discussed the great variety of prokaryotes that reportedly are capable of fixing N_2.

Agronomic Applications

Soon after Hellriegel & Wilfarth (67) published their findings in 1888 on N_2 fixation by leguminous plants in symbiotic association with rhizobia, it was recognized that inoculation of seed with rhizobia would help ensure that the leguminous roots would nodulate and fix N_2. The practice of inoculation became widespread and the production of inoculum was commercialized (23).

ROLE OF BIOLOGICAL N_2 FIXATION IN NUTRITION

Most leguminous plants are nodulated by root nodule bacteria and fix N_2 in symbiotic association with these bacteria. There are more than 13,000 described species of legumes, and they constitute a major source of protein in approximately the order listed: dry beans, dry peas (about 3 kg of each of these produced per person per year), chickpeas, broad beans, vetch, pigeon peas, cowpeas, lentils, amd lupines (9). Peanuts and soybeans are the dominant sources of cooking oils, and soybeans are used as a major food source in much of eastern Asia. Tofu and tempeh have been introduced to US markets in recent years. Areas of highest consumption of legumes as food are the former USSR, South and Central America, Mexico, India, Turkey, and Greece; Brazil produces about five times as much beans per person as we produce in the US. It is estimated that about 20% of food protein worldwide is derived from legumes. N_2-fixing nonleguminous plants are not significant sources of food.

Although diets in the United States supply only about 3% of the protein requirement as legumes, the percentage in many countries is far greater (22). As indicated, the diets in Mexico and in South and Central America are highly dependent upon legume seeds. There is a marked preference for specific legumes in these countries; some groups prefer black beans, whereas others may favor kidney beans or navy beans. The nutritional differences among these are not great, so preference appears to be based on local customs and how well the legume grows in a given area.

Leguminous plants that fix N_2 well will grow on soils that are poor in fixed nitrogen, thus making it unnecessary to add expensive nitrogenous fertilizers. However, it may be necessary to add potassium and phosphate and to lime the soil to raise the pH.

The amino acid balance of leguminous seeds commonly shows a deficiency of methionine, but combining legumes with cereal proteins usually offers a proper amino acid balance. Beans and other legume seeds thus can furnish an inexpensive, high-quality food that complements some of the deficiencies of a grain diet.

A number of toxic substances have been reported in legume seeds, and some can produce serious diseases such as neurolathyrism. Fortunately, most of the toxic materials are destroyed during cooking and hence pose little hazard.

Because of recent concerns that high fat diets may promote coronary heart disease and certain types of cancer, the public has been admonished to reduce fat intake and substitute other nutrients. As stated in *Diet and Health* (22), "Diets high in plant foods—i.e., fruits, vegetables, legumes and whole grain cereals—are associated with a lower occurrence of coronary heart disease and

cancer of the lung, colon, esophagus and stomach. . . . By using plant products (e.g., cereals and legumes) instead of animal products as sources of protein, one can also reduce the amount of saturated fatty acids and cholesterol in the diet. . . . Foods highest in dietary fiber include whole (unrefined) grains and breads made from them, legumes, vegetables, fruits, nuts and seeds." In 1985 about 18 pounds of dry beans, peas, nuts and soybeans were available per person per year in the United States.

Leguminous plants also are very important as animal feed. Alfalfa, clovers, and other legumes are grown over extensive areas as forage crops and are fed as dry hay or as silage. Maize and alfalfa are dominant as silage crops; the latter furnishes not only roughage and high quality protein but also a variety of vitamins, minerals, and other nutrients. The anaerobic ensiling process supports a rapid fermentative acidification of the plant material, and this serves to preserve its nutritional quality.

MECHANISM OF BIOLOGICAL N_2 FIXATION

Meyerhof & Burk (41) initiated biochemical investigations when they studied how N_2 fixation in *Azotobacter chroococcum* was influenced by the partial pressure of N_2 and O_2. Wilson (67) extended these studies by investigating the symbiotic N_2-fixing system of red clover and *Rhizobium trifolii*. Although the symbiotic system was difficult to manipulate, they established that half-saturation of the nitrogenase in the association occurred at a pN_2 of about 0.05 atmosphere. O_2 at high pressures cause a nonspecific inhibition of fixation. A surprising observation was that H_2 functioned as a specific, competitive inhibitor of nitrogenase. In the symbiotic system maintenance of a proper balance between fixed nitrogen and carbohydrate was necessary to support vigorous fixation of N_2 (67).

"Key" Intermediate

Arguments about the mechanism of biological N_2 fixation centered about the "key" intermediate of N_2 fixation. This was defined as the nitrogenous compound derived from N_2 that was the inorganic product of fixation that was assimilated into an organic nitrogenous compound (e.g. ammonium assimilated into glutamine or glutamic acid). Evidence for any key intermediate was scanty, although A. I. Virtanen made a strong plea for hydroxylamine in many publications (64). There is evidence that hydrazine appears as a bound intermediate (62a), but no free intermediate with a reduction level between N_2 and NH_3 has been demonstrated. When concentrated ^{15}N became available as a tracer (10), it facilitated more critical tests of potential intermediates. Exposure of various N_2-fixing organisms to $^{15}N_2$ established a pattern of assimilation suggesting that newly fixed N accumulated in glutamic acid, and

this in turn suggested NH_4^+ as the key intermediate (11). *C. pasteurianum*, in contrast to other N_2-fixing organisms, excreted NH_4^+, and when it was exposed to $^{15}N_2$ its NH_4^+ had a far higher ^{15}N concentration than any other product recovered (71). When consistent cell-free fixation of N_2 was achieved (12, 13), the product of the reaction clearly was shown to be NH_4^+ (52).

Dinitrogenase and Dinitrogenase Reductase

The achievement of consistent cell-free N_2 fixation made it feasible to purify nitrogenase. Nitrogenase consists of two proteins: an MoFe protein and an Fe protein (44, 45). These were purified essentially to homogeneity (63), and each had the same general properties when isolated from different organisms. The MoFe proteins are $\alpha_2\beta_2$ proteins of about 240,000 daltons molecular mass, whereas the Fe proteins are α_2 complexes of about 60,000 daltons molecular mass. These component proteins have been described under a variety of names (protein 1 and protein 2, fraction 1 and fraction 2, molybdoferredoxin and azoferredoxin, azofermo and azofer, etc), but we prefer the terms dinitrogenase and dinitrogenase reductase for the MoFe and Fe proteins, respectively, as these terms suggest the function of each protein (dinitrogenase binds and reduces N_2, whereas dinitrogenase reductase functions specifically to reduce dinitrogenase).

For many years scientists thought that the active site in nitrogenase centers at the Mo on dinitrogenase, but current evidence on the tertiary structure raises questions about this assumption (24a, 34a). Shah & Brill (55) demonstrated that they could dissociate and isolate an Fe- and Mo-containing center from dinitrogenase, which was designated FeMoco (for iron molybdenum cofactor). This unit could be reinserted into the apoprotein to regenerate an active dinitrogenase (51). The active site still is not defined completely, but a plausible structure of FeMoco has been presented (38). Recent crystallographic data at about 2.9 Å resolution has given a close approximation of the tertiary structure of the dinitrogenase active site (24a, 34a, 34b). The reader should refer to the original references to obtain a proper picture of the structures.

Energetics and Electron Transfer

The path of electron transfer in the nitrogenase system (Figure 1) was defined primarily by electron paramagnetic resonance (EPR) measurements at low temperatures (46). Both dinitrogenase and dinitrogenase reductase have characteristic EPR spectra that change with their oxidation-reduction state. Studies with purified nitrogenase components indicated that an external reductant (e.g. reduced ferredoxin or $Na_2S_2O_4$) passed electrons first to dinitrogenase reductase, from which they moved to dinitrogenase. The dinitrogenase then bound and reduced N_2. Each interaction of the proteins

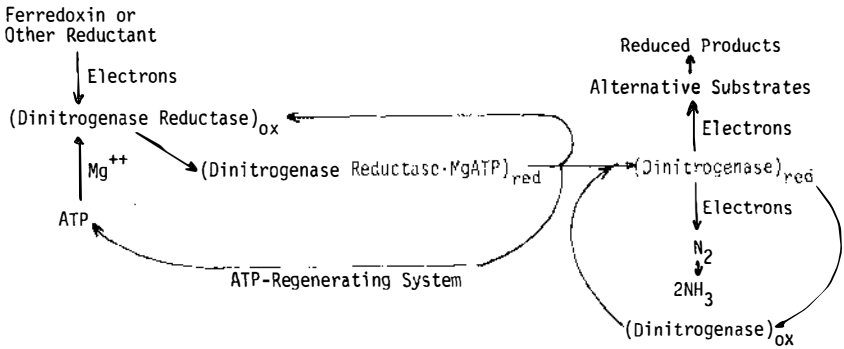

Figure 1 Electron transfer in the nitrogenase system. Electrons from ferredoxin or another reductant are passed to dinitrogenase reductase, which in turn binds 2 MgATP to yield the reduced MgATP-dinitrogenase reductase complex. This process alters the conformation of dinitrogenase reductase and drops its oxidation-reduction potential so that it can transfer an electron to dinitrogenase. Dinitrogenase and dinitrogenase reductase form a complex; one electron is passed to dinitrogenase and MgATP is hydrolyzed. Because N_2 reduction requires 8 electrons (6 electrons for N_2 and 2 electrons for the obligatory reduction of 2 protons to H_2), the dinitrogenase reductase must recycle to reduce dinitrogenase to a level adequate to reduce N_2. Only 1 electron is passed per cycle, and 2 MgATP are hydrolyzed per electron transferred.

results in the transfer of only a single electron, and as reduction of N_2 to $2NH_3$ requires 6 electrons (plus 2 electrons for obligatory reduction of 2 H^+ to H_2), the electron shuttle must operate several times before any reduction of N_2 can occur.

It was reported incorrectly that ATP inhibited cell-free fixation of N_2 (13); the ADP derived from ATP actually is the inhibitor. Therefore, the report that ATP was required as the energy source for the nitrogenase system (39) was met initially with some skepticism. Two MgATP are required for each electron transferred from dinitrogenase reductase to dinitrogenase, so a minimum of 16 MgATP are needed to drive the reaction:

$$N_2 + 8\,H^+ + 8\,e^- \rightarrow 2\,NH_3 + H_2$$

Under natural conditions, probably 20–30, rather than 16, MgATP are needed, as the process is less efficient than when optimized under laboratory conditions. The limiting step in the N_2 fixation process (Figure 1) appears to be the dissociation of the dinitrogenase-dinitrogenase reductase complex after each electron transfer (35).

As indicated, the production of H_2 that accompanies fixation of N_2 is obligatory. In the limiting case of greatest efficiency of the system, 1 H_2 (required 4 MgATP) is released for each N_2 reduced to 2 NH_3 (requires 12 MgATP). This means that 25% of the energy from MgATP is dissipated in

production of H_2. As the organism cannot block this production of H_2 (58), its only recourse is to recapture a portion of the energy by reoxidizing the H_2. In this way the organism has the potential to generate a reduced electron carrier or MgATP required in the N_2 fixation reaction. Albrecht et al (2) have shown that *hup*$^+$ *Bradyrhizobium japonicum* (soybean-infecting bacteria that contain an uptake hydrogenase) support a higher yield of soybeans than do *hup*$^-$ *B. japonicum* strains that are genetically identical except for the absence of hydrogenase.

EVOLUTION OF NITROGENASE SYSTEMS

As described below, both genetic and biochemical evidence indicates that all nitrogenases are homologous. This capability is so broadly distributed among prokaryotes that it is thought to have evolved very early and, indeed, the ability to fix N_2 might be considered a "typical" rather than an unusual attribute of prokaryotes. Regardless of the correctness of this view, it seems surprising that nature has not developed completely different enzyme systems, given both the biological importance of the enzyme's activity and the relatively slow turnover rate of the known nitrogenases.

There is little doubt that the common ancestor of all known nitrogenases was the product of substantial evolution. The α and β subunits of dinitrogenase, which make up the $\alpha_2\beta_2$ tetramer, are homologous to each other, reflecting an ancient gene duplication. These proteins are also homologous to another protein involved in nitrogen fixation, the NifNE protein, which functions in the synthesis of FeMoco (6, 62). (The term *nif* refers to genes involved in nitrogen fixation, and NifNE to the protein products of the *nifN* and *nifE* genes.) Future analysis of the structure-function relationships within these two complexes should provide some interesting insights into the evolution of protein function.

Similarity of Nitrogenases From Diverse Sources

N_2-fixing systems from diverse organisms seem to be very similar, i.e. they all require dinitrogenase and dinitrogenase reductase, all require a strong reductant, and all require MgATP as an energy source. The homology among the dinitrogenases and dinitrogenase reductases from diverse N_2-fixing organisms is very striking. Detroy et al (17, 18) first showed that the dinitrogenase reductase from one N_2-fixing organism could be combined with the dinitrogenase from another organism to reconstitute a functional nitrogenase. This observation was extended (20) to preparations from 8 N_2-fixing organisms; these furnished 64 possible dinitrogenase-dinitrogenase reductase combinations, 8 of which were homologous. Among the 56 nonhomologous crosses,

85% produced active nitrogenases. These represented proteins from aerobic, anaerobic, facultative, photosynthetic, free-living, and symbiotic N_2-fixing organisms.

There are variations on this theme, however. Some dinitrogenases have a different subunit composition and are devoid of molybdenum. In these "alternate" dinitrogenases molybdenum may be replaced by vanadium, and in others it is proposed that iron replaces the molybdenum (5).

Clearly, different nitrogenase systems have developed slightly different mechanisms for addressing the maturation and functioning of the enzyme. The fundamental similarity of the underlying biochemical mechanisms is, however, more striking than the differences.

The ability to fix N_2 has been found in a broad range of bacteria, including representatives of both the archaebacteria and eubacteria (70). This diversity of organisms, when contrasted with the remarkable conservation of nitrogenase itself, immediately suggests the possibility of horizontal transfer. The data, while not unequivocal, suggest otherwise. Generally speaking, a phylogeny of diazotrophs based on their 16S RNA yields a tree similar to one based on the sequence of *nifH* (30, 70), which suggests that if horizontal transfer did occur it was between relatively related organisms.

Other arguments against horizontal transfer based on *nifH* sequences appear less strong. Although it is true that *nifH* sequences have the same GC content as the rest of the chromosome and employ "typical" codon usage (59, 70), this merely suggests that there will be a strong selection for each of these features in the case of a gene that must be so highly expressed (57). As our data bases grow, it will be interesting to see if the same points will hold for the less abundantly expressed *nif* genes. Finally, phylogeny arguments based on *nifH* are complicated by the organisms that have multiple *nifH* sequences (65). Some of these encode dinitrogenase reductases for alternate nitrogenase systems. Others may not even express a functional product, but their presence makes it difficult to choose the appropriate gene for the phylogenetic analysis.

The structural genes for dinitrogenase and dinitrogenase reductase are much more conserved than the other *nif* genes; this is revealed most directly by sequence comparison, but also by the relative ability to detect the genes by hybridization with heterologous gene probes. It seems plausible that the first two proteins have a number of biochemical features that must be maintained if they are to remain biochemically functional. These include domains for protein-protein interactions to form their own multimers, domains for binding the various necessary metal clusters and for interactions with each other, and domains for binding with the low potential electron donor and with the various gene products involved in their maturation. In contrast, it is likely that the other *nif* gene products require a much smaller number of domains for their

function, thereby freeing the genes to evolve more rapidly. In the case of other *nif* genes, examples of gene splitting (66) and gene fusion (15) suggest significant plasticity in their gene products.

Adaptation of Nitrogenase to Different Organisms and Environments

The fundamental problem facing any diazotroph is how to protect its extremely O_2-labile nitrogenase proteins from O_2. Secondary problems are the availability of ATP and securing a supply of low potential electrons and a source of metal for the active sites of dinitrogenase.

Anaerobic diazotrophy removes the problem of O_2-sensitivity, and fermentation provides an abundance of reductant for N_2 fixation. To solve the problem of sufficient ATP levels to support N_2 fixation, the "typical" free-living diazotroph oxidizes organic compounds, but alternative sources include photosynthesis, H_2 oxidation, and chemolithotrophic metabolism (32).

Given the O_2 lability of nitrogenase, the growth of any diazotroph in the presence of O_2 is beset with problems. Nitrogenase must be protected from the O_2, and nitrogenase and respiration will compete for reductant. The advantage of aerobic N_2 fixation is that respiration provides an abundance of energy. The general strategies for fixing N_2 in the presence of O_2 require physical protection of the enzyme. In many cases, this involves the construction of a specialized cell or cell compartment. Such compartments include the heterocysts of the cyanobacteria discusscd below as well as thc nodule that is characteristic of most symbiotic diazotrophs. In the nodule, the level of free O_2 is effectively controlled by the presence of leghemoglobin. For energy, symbiotic N_2-fixers rely on photosynthate supplied by their host plant, typically in the form of carbohydrates and organic acids.

As photosynthetic organisms, the cyanobacteria (or blue-green algae) liberate O_2 and still maintain an active nitrogenase (28). Most commonly, they accomplish this by producing heterocysts, which are specialized cells that differentiate to lose their photosynthetic capacity to generate O_2 while gaining the capability to fix N_2. The vegetative cells in the filament maintain active photosynthesis and transfer photosynthate to heterocysts to supply the energy needed by the heterocysts to fix N_2. Seldom are more than 10% of the cells differentiated into heterocysts, although in an association between a small water fern *(Azolla)* and a blue-green alga *(Anabaena azollae)* about 30% of the cells in the alga may be heterocysts (48). This productive *Azolla-Anabaena azollae* system has been used for centuries to add fixed nitrogen in rice culture in southeast Asia. In another adaptation certain filamentous marine cyanobacteria have adopted a diurnal cycle that alternates photosynthesis in the day with fixation of N_2 at night when there is no photosynthetic production of O_2 (42). These systems furnish models for those who are attempting to

introduce the O_2-sensitive nitrogenase system into O_2-generating photosynthetically active plants.

Although the aerobes in the genus *Azotobacter* are among the most intensively studied organisms both biochemically and genetically, the basis for their O_2 tolerance is not well understood. When purified, their nitrogenase components are as O_2-labile as those from any other source (54). Shethna found that very gentle cell breakage allowed the isolation of the nitrogenase activity in a large complex (56). This complex showed substantial O_2-tolerance in vitro and appeared to contain other proteins including an iron-sulfur protein, often termed the Shethna protein. Unfortunately, these observations have not been actively pursued. Another basis for the O_2-tolerance of the azotobacters might well be their extremely high respiration rate, which potentially could reduce the O_2 concentration within the cell (49).

Given the amount of dinitrogenase necessary to sustain growth on N_2, it is not surprising that the supply of metals for the active site of the enzyme might occasionally be limiting. Those diazotrophs that possess more than one nitrogenase respond to such limitation by expressing their alternate nitrogenases. Although the actual mechanism of the response is not entirely clear, apparently the products of a set of regulatory genes recognize metal availability and activate the set of genes appropriate to that metal (5). Another layer of regulation, apparently involving the various dinitrogenase reductases themselves, also serves to further shut down expression of the "nonfunctional" nitrogenases (33).

ORGANIZATION OF GENES ENCODING NITROGENASE SYSTEMS

Linkage of Known nif *Genes*

Whereas the genes encoding dinitrogenase and dinitrogenase reductase have been cloned and sequenced from a wide variety of organisms, the other *nif* genes have been studied in only a few organisms. In these cases, the *nif* genes have been found in one or two gene clusters (15). Again, the presence of the alternate nitrogenases complicates the story, especially because they utilize at least some of the proteins involved in maturation of the Mo-containing dinitrogenases.

Cotranscription and Coregulation of nif *Genes*

While substantial clustering of *nif* genes is the rule, there is little consistency in their transcriptional organization (15). Presumably, the cotranscription of different genes allows their expression to be regulated identically in timing

and in amount, although different types of post-transcriptional regulation certainly also could affect synthesis of the protein product (25).

In the case of the *nif* genes, however, it is unclear what selections have created the transcriptional units that are found. For example, differential timing of expression of *nif* transcripts has not been demonstrated other than of the regulatory genes themselves. The data on the actual accumulation level of most of the *nif* products is incomplete, so an explanation of cotranscription based on comparable product synthesis cannot be tested. Our current limited understanding of *nif* gene product function also makes it difficult to fully rationalize the transcriptional organization on this basis. Certainly the genes encoding the major components of nitrogenase tend to be cotranscribed, but *B. japonicum* (1) and others provide exceptions. That other genes are present in the same transcript as the structural genes for nitrogenase is also puzzling (3).

Genome Rearrangements in nif *Regions*

Genome rearrangements, including inversions and amplifications, have been identified in a number of bacteria, but two of the best-studied cases of site-specific deletions are in the *nif* operon (26, 27). Deletions are unique among rearrangements because they are irreversible. Thus, it is not surprising that the examples of *nif* deletions have only been detected in the "terminally differentiated" heterocyst cells of *Anabaena*. In these cells, two deletion events must occur for the proper transcription of two different *nif* operons (26, 27). These events take place during differentiation and eventually allow these cells to produce functional nitrogenase. Such deletions would not be an acceptable regulatory mechanism for growing cells, but it is unclear why they should be useful to the terminally differentiated cells in which they occur.

The aforementioned duplications of *nifH* in some organisms also might play a role in the physiology of the cell, though that role currently is unclear.

CONTROL OF NITROGENASE

N_2 is an unattractive nitrogen source, largely because of the high activation energy required for its reduction. This accounts for the high energy demand for the process and may contribute to the relative "slowness" of nitrogenase itself and the need to accumulate large amounts of nitrogenase to supply the cell's nitrogen requirements. The implications of these constraints are twofold. First, cells are "reluctant" to derepress the *nif* system until it is very clear that there are no better nitrogen sources. Then, after the system has been elaborated, the presence of a better nitrogen source, or changes in the environment that preclude N_2 fixation, must be addressed by shutting down

N_2 fixation. These constraints have given rise to various levels of regulation that differ in both the timing and the degree of their effects.

Transcriptional Control by "Global" Regulators

All diazotrophs must determine if N_2 is their best available nitrogen source, and apparently this question is resolved predominately by the global nitrogen regulatory system (the products of the *ntr* genes) (50). In contrast to most catabolic systems, the substrate N_2 is not required for induction. As N_2 is normally present, the cell instead seeks other available nitrogen sources. This is accomplished by elements of the *ntr* system that appear to use levels of such metabolically central compounds as glutamate and glutamine to determine if nitrogen is limiting (50). It is curious, and often ignored, that the interval between the onset of starvation and the induction of *nif* expression is protracted (usually one to two hours). It is unclear whether this is the time required to deplete sufficiently all available nitrogenous compounds within the cell or whether it is a deliberate regulatory device to preclude derepression of nitrogenase when its presence would be unnecessary.

The actual mechanism of the *ntr*-mediated regulation of *nif* is rather well understood, at least in the case of *K. pneumoniae*. The *ntr* gene products signal the activation of the NtrC protein by a phosphorylation mechanism (34), which follows the paradigm of other two-component regulatory systems (61). This activated NtrC, in concert with the sigma factor RpoN, activates transcription of *nifLA,* with *nifA* encoding the *nif*-specific regulator discussed below. Because this regulation involves activation of a regulatory transcript, it does not provide an obvious mechanism for shutting down *nif* expression rapidly when a better nitrogen source becomes available. Other diazotrophs appear to use slight variations on this theme; often the NtrC homolog is not the general transcription regulator that it appears to be in *K. pneumoniae*.

In those organisms that regulate *nif* expression in response to O_2, the effect of O_2 seems to be to regulate the expression of the *nif*-specific transcriptional activator. An obvious candidate for such an effector would be the protein product of the *fnr* gene, which is known to activate the expression of a number of anaerobically regulated genes (59). Unfortunately, experiments to test the involvement of the *fnr* product in *nif* regulation were performed in a heterologous background, so the negative results obtained must be accepted with caution (31).

An entirely different proposal for anaerobic regulation posits that the degree of supercoiling reflects the aerobicity of the cell and that, in turn, it directly affects the functionality of specific promoters and transcription factors (69). The basis of the model is the effect of specific inhibitors of gyrase in blocking anaerobic gene expression. This model has received much support, largely

through identical experiments performed in different organisms. However, an entirely different mode of analysis of anaerobic gene expression has found no such "anaerobic-specific" effects of supercoiling (14), so the issue should be considered undecided until new experimental approaches are tried.

nif-*Specific Transcriptional Control*

If the global systems described above are capable of determining both nitrogen starvation as well as anaerobiosis, why should the cell have one or more *nif*-specific regulatory proteins? First, a specific factor probably is useful for coordinating the expression of the numerous *nif* operons. Second, nitrogenase is a slow enzyme and when the cell is in nitrogen-excess, a minimal level of nitrogenase is advantageous; under nitrogen-limiting conditions, however, the cell needs massive amounts of the nitrogenase proteins. Thus, a single level of regulation might not be sufficient to provide the huge difference seen in nitrogenase activity under these two conditions (10^5-fold at least). Finally, at least in some organisms, the *nif*-specific regulators provide another level for sensing and responding to the nitrogen and O_2 status of the cell. Since these are *nif*-specific, their useful range can be tuned to rather lower levels of O_2 or fixed nitrogen than those typically addressed by a global system. Such a *nif*-specific response also supports a much more rapid transcriptional response than would be provided by the global systems.

In studied organisms, the *nif*-specific activator is either NifA or its homolog; it apparently acts by binding to an "Upstream Activator Sequence" (7) and facilitates the isomerization of RNA polymerase (including the RpoN sigma factor) from a closed to an open complex (43). In some diazotrophs, another regulatory factor, NifL, appears to interfere with the activation by NifA when O_2 or fixed nitrogen is present (40). The mechanism by which NifL senses these compounds is completely unknown, as is the precise way it blocks NifA function. In other diazotrophs, the NifA homolog appears to be an O_2-sensor itself (21).

Posttranslational Control of Nitrogenase

Although transcriptional regulation is important, it necessarily effects a slow response to a change in the environment. Some diazotrophs have developed posttranslational systems that allow a much more rapid turn-off of the energy-demanding nitrogenase system. One of the most thoroughly studied of these is covalent modification of the dinitrogenase reductase by ADP ribosylation.

Rhodospirillum rubrum is a photosynthetic purple nonsulfur bacterium that is capable of N_2 fixation. Although *R. rubrum* yielded N_2-fixing cell-free preparations (52), consistently active preparations were difficult to obtain. Ludden & Burris found (36) that the organism possessed an enzyme that would

convert inactive to active preparations. Subsequently it was observed (37) that *R. rubrum* also had an inactivating enzyme. Thus it had the capability to switch nitrogenase activity on and off.

The system was explored in detail (37) and is depicted in Figure 2. The activation-inactivation affects dinitrogenase reductase, apparently without participation of dinitrogenase. The enzyme that inactivates nitrogenase activity, termed dinitrogenase reductase ADP-ribosyl transferase (DRAT), uses NAD as a source of an ADP-ribosyl group, which it transfers to a particular arginine on one of the two subunits of dinitrogenase reductase. Nicotinamide is released and the reaction can be followed with ^{32}P-labeled NAD (51).

Dinitrogenase reductase activating glycohydrolase (DRAG) removes the ADP-ribosyl group from the arginine residue, and thus restores activity to dinitrogenase reductase. This change also can be followed on gels, as the band from the modified subunit loses its ADP-ribosyl group and regains its original mobility.

The DRAT-DRAG system will turn off nitrogenase activity in response to addition of NH_4^+ (24), and, in some organisms, to the presence of O_2. In some photosynthetic organisms it also responds to darkness, because photosynthetic energy is required for fixation of N_2. In microaerobic *Azospirillum* spp. the system responds to anaerobiosis because low respiration is necessary to support N_2 fixation.

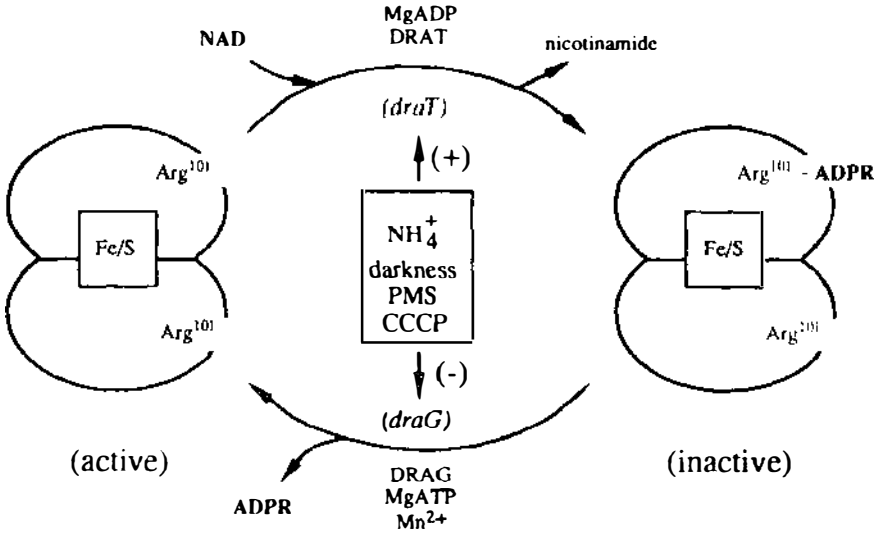

Figure 2 Control of dinitrogenase reductase activity by ADP-ribosylation as depicted by Ludden & Roberts (37). ADPR, ADP-ribose; PMS, phenazine methosulfate; CCCP, chlorocarbonylcyanide phenylhydrazone.

Not all N_2-fixing organisms possess a posttranslational regulatory system, but some that do appear to use mechanisms other than ADP ribosylation. An interesting case is presented by the *Azospirillum* spp. The azospirilla are a group of microaerobic N_2-fixing organisms that are described as associative N_2-fixing bacteria. They do not function in a tight symbiosis as do rhizobia in the nodules of leguminous plants, but rather they grow on the roots (and sometimes within the roots) and utilize excreted or sloughed material as their energy source in support of N_2 fixation. *Azospirillum brasilense* and *Azospirillum lipoferum* have well-developed DRAT and DRAG systems for the reversible control of N_2 fixation (72). However, *Azospirillum amazonense* from the same genus lacks the system according to the following criteria: it lacks DNA homologous to *draT* and *draG* probes, it lacks detectable DRAG and DRAT antigens, and it does not alter the mobility of dinitrogenase reductase upon switch-off of activity.

CONCLUSION

The stability of the N_2 bond has dictated constraints on the enzymatic and biochemical mechanisms of biological N_2 fixation. Different microorganisms, many of them serving critical functions in world agriculture, have developed a fascinating range of adaptations within these constraints. One hundred years after the clear demonstration of biological N_2 fixation, we still are seeking to understand and to utilize fully this important process.

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