NITROGEN FIXATION BY PHOTOSYNTHETIC BACTERIA

(Rhodospirillum rubrum, Rhodopseudomonas capsulata, glutamine, nitrogenase)

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SUMMARY

Biological nitrogen fixation is not only essential for world nitrogen balance but it is also an alternative to expensive commercial fertilizer for crop production. To achieve the maximum utilization of this natural process, an understanding of the mechanism of N_2 reduction and its regulation is being sought. The photosynthetic bacteria, in particular members of the Rhodospirillaceae, *are attractive organisms for genetic and biochemical analyses of nitrogen fixation. Characterization of mutants of these bacteria derepressed for synthesis of the nitrogenase complex in the presence of ammonium salts supports a critical role for glutamine and α-ketoglutarate in the regulation of synthesis. In addition, a mechanism exists for activity control by covalent modification of one of the protein components of the complex. The signal for modification and the extent to which this control mechanism occurs in other diazotrophs are under investigation.*

INTRODUCTION

Biological nitrogen fixation, the reduction of dinitrogen to ammonia, is a procaryotic process. Interest in this process has been rekindled by the economic need to reduce costs of fertilizer production and by the development of new genetic engineering technology making the creation of novel nitrogen-fixing organisms possible. This renewed attention has intensified the investigation of the physiology, enzymology, and genetics of nitrogen fixation. However, its regulation remains to be elucidated, especially in the purple photosynthetic bacteria, where controls not present in other diazotrophs may be functioning. In the following pages, the evidence for such controls will be presented, as well as some suggestions as to their mechanisms.

DISCOVERY AND ENZYMOLOGY

Discovery of the capacity of the photosynthetic bacteria to fix dinitrogen resulted from observation of light-dependent production of molecular hydrogen (GEST and KAMEN 1949). In 1949 Kamen and Gest found that hydrogen production by cultures of *Rhodospirillum rubrum* was prevented by the presence of N_2 or excess ammonium salts. They interpreted these results as evidence that a nitrogenase complex was responsible for H_2 formation (KAMEN and GEST 1949). This interpretation was confirmed (GEST et al. 1950) and extended to almost all members of the *Rhodospirillaceae* (LINDSTROM et al. 1951).

A general diagram of nitrogen fixation by a photosynthetic bacterium growing photoheterotrophically is shown in Figure 1.

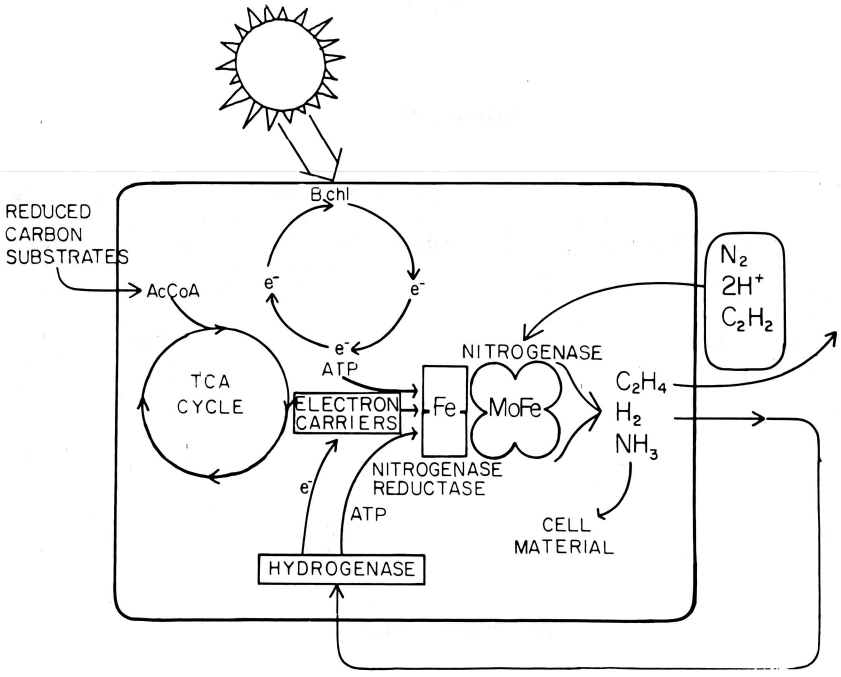

FIGURE 1. Diagrammatic representation of nitrogen fixation by a photosynthetic bacterium growing photoheterotrophically. AcCoA, acetylcoenzyme A; Bchl, bacteriochlorophyll.

In this process, light or, in nature, sunlight is used to generate energy by cyclic photophosphorylation. Reducing power is obtained by the oxidation of reduced carbon substrates provided in the medium (not from water as in higher plants or cyanobacteria), the most effective substrates being TCA cycle intermediates (SCHICK 1971; HILLMER and GEST 1977). When synthesis of nitrogenase proteins is derepressed, electrons are supplied to the enzyme complex via electron carriers, perhaps spe-

cific to this system, such as ferredoxin or flavodoxin.

Two proteins have been identified in all systems as necessary for substrate reduction (MORTENSON 1978), an iron-containing protein (nitrogenase reductase) and a molybdenum-and-iron-containing protein (nitrogenase). In *R. rubrum* the two corresponding proteins are quite similar in composition and size to those from other diazotrophs, with the Fe protein being a dimer of 61,500 M.W., and the MoFe protein a tetramer of 230,000 M.W. (LUDDEN and BURRIS 1978; NORDLUND et al. 1978). With an adequate supply of ATP, Mg^{2+}, and reducing power, nitrogenase proteins reduce dinitrogen to ammonia and, simultaneously, reduce protons to hydrogen gas. Subsequently, ammonia is assimilated into glutamine via the energy-consuming reaction mediated by glutamine synthetase (JOHANSSON and GEST 1976; BROWN and HERBERT 1977a and b), and the amide nitrogen is then available for the biosynthesis of cellular materials.

In the absence of N_2 or other reducible substrates, protons are the exclusive substrate, and copious amounts of hydrogen gas are formed (KAMEN and GEST 1949; HILLMER and GEST 1977). Although less hydrogen is produced during N_2 fixation (KELLEY et al. 1977), it still represents a considerable energy loss (MORTENSON 1978). Some of this energy may be recaptured through the oxidation of hydrogen by an uptake hydrogenase (SCHUBERT and EVANS 1976; KELLEY et al. 1977).

Finally, the remaining substrate shown in Figure 1, acetylene, is reduced to ethylene by the nitrogenase system (DILWORTH 1966; SCHOLLHORN and BURRIS 1967). This capacity provides a simple, accurate method of assaying the activity of the enzyme complex and has rapidly advanced our understanding of N_2 fixation and the extent of its occurrence.

The activity of the nitrogen-fixing complex in most diazotrophs is inhibited by ADP, carbamyl phosphate, and H_2 both *in vivo* and *in vitro* (MORTENSON 1978). Neither ammonia, the product of N_2 fixation, nor other N-containing compounds resulting from the initial steps of ammonia incorporation appear to affect activity (MORTENSON 1978). However, in whole cells of the photosynthetic bacteria, a reversible inhibition of activity by ammonium salts was seen (GEST et al. 1950; NEILSON and NORDLUND 1975; ZUMFT and CASTILLO 1978) which was not demonstrable in crude extracts or purified preparations of the nitrogenase complex (NEILSON and NORDLUND 1975).

Other anomalies of the nitrogen-fixing system of the photosynthetic bacterium *R. rubrum* were observed in early studies. The time course of reductions catalyzed in cell-free extracts by nitrogenase were nonlinear and showed a lag. In addition, reproducible activity was difficult to obtain (MUNSON and BURRIS 1968). These unusual properties provided the impetus for a more detailed biochemical analysis to determine whether the dinitrogen reducing system in these organisms was fundamentally different from others for which data were available.

In 1976, Ludden and Burris demonstrated that a component

isolated from chromatophores could increase nitrogenase activity in crude extracts of *R. rubrum*. Independently, Nordlund et al. (1977) obtained essentially similar results. Pretreatment of crude extracts or purified Fe protein with the component, called "activating factor" (AF), in the presence of ATP and a divalent cation, rendered the time course of reductions linear and eliminated the lag. In addition, AF was shown to be trypsin-sensitive and O_2-labile (LUDDEN and BURRIS 1976). Information obtained during the characterization of the activating system explained many of the earlier problems encountered in enzymological studies with this system.

The molecular nature of the inactivation and reactivation of the Fe protein was pursued by Ludden and Burris (1978). Their investigation of the physical and chemical properties of purified Fe protein led to the discovery of phosphate, a pentose sugar and an adenine-like compound present in the inactive protein. The data indicated a composition of protein/phosphate/pentose/"adenine" of 1:2:2:2. By comparing inactive and active Fe protein, these workers obtained evidence for the removal of the adenine-like compound during activation (LUDDEN and BURRIS 1979, Figure 2). *In vitro* inactivation has not yet been achieved

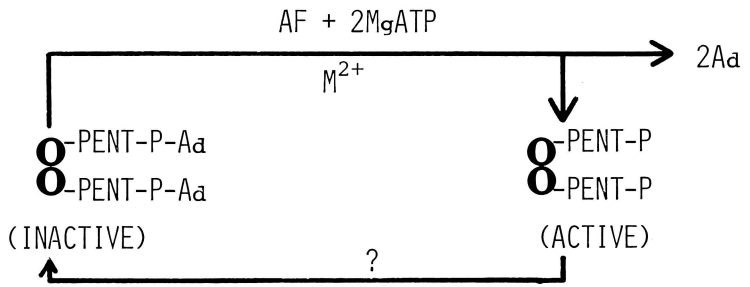

FIGURE 2. Hypothesis of activation and deactivation of the Fe protein of *R. rubrum*. Pent, pentose; P, phosphate; Ad, adenine-like compound; O, subunit of Fe protein.

(LUDDEN and BURRIS 1979), leaving the question of the physiological role of this activity regulation unanswered. However, this novel control process may represent a generalized mode of regulation in the purple photosynthetic organisms (ZUMFT and CASTILLO 1978; YOCH 1979).

REGULATION OF NITROGENASE SYNTHESIS

Based on physiological and enzymological studies of N_2 fixation, the synthesis of nitrogenase in the photosynthetic bacteria appears to be regulated like that of other diazotrophs (ORMEROD et al. 1961). Ammonium salts and high levels of O_2 appear to repress synthesis of the proteins involved (WALL, unpublished results). Although little is known concerning the mechanism of O_2 repression, considerable information and many theories have been generated to explain the effect of ammonia

(DILWORTH 1974; MORTENSON 1978).

The major pathway of ammonia assimilation in N_2-fixing organisms is the enzyme couple glutamine synthetase/glutamine (amide):2-oxo-glutarate amidotransferase[glutamate synthase (MEERS et al. 1970)] catalyzing reactions (1) and (2) respectively (NAGATANI et al. 1971). Although glutamate dehydrogenase [catalyzing reaction (3)] is also present in many of these bacteria, it appears to be of importance for assimilation only when ammonia levels are quite high (MEERS et al. 1970).

$$NH_3 + \text{L-glutamate} + ATP \longrightarrow \text{L-glutamine} + ADP + P_i + H_2O \quad (1)$$

$$\text{L-glutamine} + \alpha\text{-ketoglutarate} + NAD(P)H + H^+ \longrightarrow 2\,\text{L-glutamate} + NAD(P)^+ \quad (2)$$

$$\alpha\text{-ketoglutarate} + NH_4^+ + NAD(P)H \leftrightarrow \text{L-glutamate} + NAD(P)^+ \quad (3)$$

Of these enzymes, glutamine synthetase (GS) is the target for regulation in response to cellular nitrogen metabolism. Changes in enzyme activity occur in response to ammonia concentration through an elaborate cascade system of covalent modification (GINSBERG and STADTMAN 1973; WOHLHUETER et al. 1973). As ammonia levels decrease, covalent modification of GS is decreased and GS activity and protein increase. In addition, the synthesis of other enzymes capable of supplying the cells with usable nitrogen, such as amino-acid-degrading enzymes, appear to be under the same control (MAGASANIK 1977).

A regulatory model was proposed (MAGASANIK 1977; TYLER 1978) in which glutamine synthetase functioned directly as a genetic element controlling transcription of its own structural gene and that of other genes involved in providing nitrogen for cell growth. A major feature was that the regulatory effects mediated by GS were a consequence of the degree of covalent modification. Evidence for this model was provided by the isolation of two types of mutants believed to lie within the structural gene for GS: 1) those which eliminated the synthesis of GS and greatly lowered synthesis of other proteins under nitrogen control and 2) those which resulted in constitutive high-level synthesis of all these proteins (MAGASANIK et al. 1974). Additional support was provided by the regulatory effects of purified GS on the *in vitro* transcription of genes for histidine degradation (TYLER et al. 1974).

As a logical extrapolation, this model of control was extended to the regulation of nitrogenase (STREICHER et al. 1974). To test this model, Streicher et al. (1974) constructed special strains containing the presumed GS mutations described above and demonstrated that nitrogenase synthesis was controlled in a manner similar to that of other nitrogen-metabolism proteins. More evidence for GS involvement in nitrogenase regulation was provided by the observation that the GS inhibitor methionine sulfoximine brought about derepression of nitrogenase biosynthesis (GORDON and BRILL 1974).

Recently, observations which did not fit this model were reported. First, the correlation between covalent modification

of GS and the synthesis of GS and nitrogen-metabolizing enzymes was found to be lacking in a number of experiments (SENIOR 1975; TYLER 1978). Second, evidence that GS cannot be the only regulatory factor for its own synthesis was demonstrated in *Salmonella* by the discovery of a regulatory gene, *glnF*, the protein product of which is essential for GS synthesis (GARCIA et al. 1977). More recently a third protein was shown to be necessary for regulation of GS synthesis, the product of the *glnG* gene in *Escherichia coli* (PAHEL and TYLER 1979) or *glnR* in *Salmonella* (KUSTU et al. 1979). This third gene, *glnR*, maps very close to the structural gene for GS, *glnA*. Because the original model for the involvement of GS in regulation of nitrogen metabolism was based on the phenotype of mutants believed to map in *glnA*, the possibility arises that these lesions may lie in *glnR* rather than in *glnA*.

These results bring the role of GS in regulation into question (PAHEL and TYLER 1979; KUSTU et al. 1979). Therefore, a new model for the regulation of GS synthesis was proposed by Kustu et al. (1979) which takes into account the functioning of the recently discovered regulatory proteins (see Figure 3).

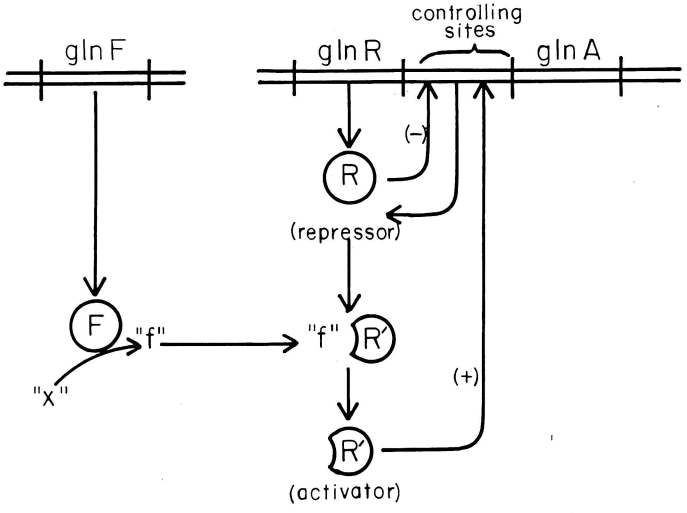

FIGURE 3. Model for control of *glnA* transcription by the *glnR* and *glnF* products (taken from KUSTU et al. 1979).

Like the earlier model, this one proposes that the regulation occurs at transcription. Kustu et al. (1979) suggested that the protein product of the *glnR* gene can exist in two conformations: R, which is a repressor for *glnA* transcription, and R', which is an activator, facilitating transcription. The product of *glnF* leads to the conversion of R to R' in response to nitrogen deficiency, perhaps indirectly by catalyzing the formation of a

low-molecular-weight signal, "f," which interacts with R. These workers also suggest that R' may be involved in stimulating the transcription of other genes under nitrogen control as well.

The limited information available concerning the regulation of GS and nitrogenase synthesis in the photosynthetic bacteria is compatible with this latest model. The enzymes responsible for ammonia assimilation in the phototrophs were only recently examined. In *Rhodopseudomonas capsulata*, the GS/glutamate synthase enzyme couple is the primary pathway for ammonia assimilation (JOHANSSON and GEST 1976) and there is evidence for regulation of GS activity by covalent modification (JOHANSSON and GEST 1977). Surprisingly, activity of the enzyme most often associated with assimilation, glutamate dehydrogenase, was undetectable in *R. capsulata* (JOHANSSON and GEST 1976).

Two mutants of photosynthetic bacteria have been reported to be derepressed for nitrogenase synthesis and activity in the presence of ammonium salts: 1) a glutamate auxotroph of *R. rubrum* defective for glutamate synthase activity (WEARE 1978) and 2) a glutamine auxotroph of *R. capsulata*, G29, lacking detectable GS activity (WALL and GEST 1979). Figure 4 shows a comparison between H_2 production mediated by nitrogenase from the *R. capsulata* derepressed mutant G29 and the wild-type strain B10. The wild-type culture produced no gas when ammonium salts were present in the medium, while the mutant G29 produced the same quantity regardless of the ammonia concentration (see also Table 1). Characterization of this mutant as well as the *R. rubrum* mutant confirmed that ammonia is not the direct signal for repression and that the assimilatory enzymes or their products might be involved.

During experiments with the *R. capsulata* mutant G29, it became clear that in minimal medium containing ammonium salts a larger amount of glutamine (> 7 mM) was necessary to support maximum growth of the cultures than the amount of supplement required for other auxotrophs (< 0.1 mM). Glutamine appeared to be the sole nitrogen source, not simply a growth supplement. These results confirmed that ammonia is assimilated primarily through a single pathway which is blocked in this mutant. The consequence of not clearly grasping this information was that observations of derepression were usually made after glutamine was exhausted from the culture medium. When care was taken to maintain an excess supply of glutamine, no derepression of nitrogenase was observed either by visible gas production or by acetylene reduction assays (WALL, unpublished results; see Table 1).

At present, 18 different Gln^- strains of *R. capsulata* have been isolated and all are derepressed for nitrogenase synthesis and activity. All are linked using the genetic exchange system unique to this species, the "gene transfer agent" (MARRS 1974). This vector randomly packages and transfers a linear, double-stranded piece of DNA of molecular weight 3×10^6 (SOLIOZ and MARRS 1977). Therefore, linkage determined by a vector of this size indicates a very tight clustering of mutations on the genetic map. However, because complementation analysis is not

TABLE 1. Nitrogenase Activities of *R. capsulata* B10 (wild type) and the Gln^- Mutant G29.

Strain	Nitrogen Source[a]	Nitrogenase Activity[b]
B10	Gln (5 mM)	2.06
B10	Gln (5 mM) + NH_4^+	<0.01
B10	Gln (15 mM)	<0.01
G29	Gln (5 mM)	2.17
G29	Gln (5 mM) + NH_4^+	3.00
G29	Gln (15 mM)	<0.01

[a] The minimal medium lacked ammonium salts and was supplemented with 7.5 mM $(NH_4)_2SO_4$ and/or 5 mM glutamine or with 15 mM glutamine (Gln) alone.

[b] Acetylene reduction is expressed as micromoles of ethylene per hour-milligram (dry weight). Cells were grown photosynthetically to the stationary phase in glass syringes. A 1-ml portion of this culture was directly injected into a 12-ml stoppered vial which had previously been flushed with argon. After injection of 0.5 ml of acetylene, the vials were incubated at 33°C for 1 h in saturating light (about 6,500 lux) with occasional shaking. Samples were then placed in darkness until estimation of ethylene by gas chromatography (Hewlett-Packard model 402 analyzer fitted with a Porapak R column operating at 75°C).

available in this region of the chromosome, it was not possible to establish whether the mutations lie in one or two genes, perhaps corresponding to *glnR* and *glnA* of *Salmonella* (Figure 3). In the *Salmonella* system, lesions in *glnR* do not require glutamine for growth and those in *glnF* cannot be derepressed for GS synthesis or synthesis of other genes under nitrogen control (KUSTU et al. 1979). Because the Gln^- *R. capsulata* strains are all tight auxotrophs and because nitrogenase can be derepressed, these mutations are most likely in the structural gene for GS, *glnA*. To confirm this possibility, temperature-sensitive Gln^-

FIGURE 4 (opposite page). Effect of NH_4^+ on H_2 formation by *R. capsulata* B10 (wild type) and Gln^- mutant G29. Growth vessels are modified glass syringes; the usual external end of the hollow plungers was cut off and the plunger inverted. At zero time, each syringe was completely full of inoculated medium (50 ml total volume, including 5 ml of inoculum; the latter consisted of stationary phase cells grown photosynthetically in minimal medium minus ammonium sulfate plus 5 mM Gln). In all instances, the medium contained 30 mM DL-lactate, 5 mM Gln, as well as mineral salts and vitamins for minimal medium; where indicated, 7.5 mM ammonium sulfate was also present. The syringes were illuminated with saturating light at 30°-33°C and the photograph taken after 8 hours of incubation. Gas accumulated in the hollow plungers is almost pure H_2 (HILLMER and GEST 1977).

strains are now being sought.

If indeed all the 18 Gln^- mutants have lesions in *glnA*, it seems unlikely that all 18 defective GS proteins would have retained the ability to efficiently repress and/or derepress nitrogenase. Although this possibility has not yet been eliminated by the results, it would seem more likely that a direct or indirect product of GS activity is responsible for the altered regulation. Figure 5 shows a modified form of Kustu's

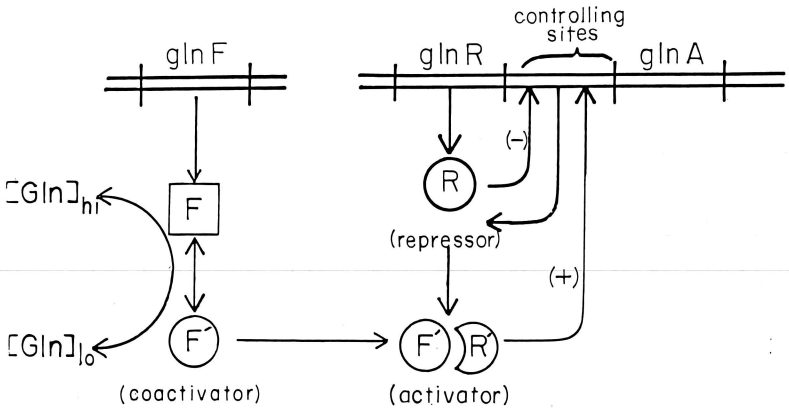

FIGURE 5. Modified scheme for control of *glnA* transcription (and other genes under nitrogen control, particularly the nitrogenase enzyme complex).

model (KUSTU et al. 1979) which readily accommodates the information available. In this scheme, glutamine concentration is the signal for nitrogen control. Thus the protein product of *glnF* can exist in two conformations and is converted from F to F', a coactivator, when the glutamine concentration drops below a critical level. F' can then interact with R, the repressor, forming an F'R' activator complex. Among alternative scenarios is the possibility that F itself is a coactivator existing in only one conformation and that F is prevented from interacting with R by high levels of glutamine. When the glutamine concentration drops below a critical level, R is free to interact with F, forming an FR' activator complex which stimulates transcription.

While the roles of other metabolites are not included in this scheme, the derepressed *R. rubrum* mutant lacking glutamate synthase activity (WEARE 1978) supports an involvement of α-ketoglutarate in nitrogenase regulation. Evidence that both glutamine and α-ketoglutarate levels are important physiological signals for regulation of GS activity was obtained from continuous culture experiments with *E. coli* (SENIOR 1975). However, the actual signal may be a metabolite derived from these compounds.

In the past, the repression of synthesis of enzymes involved in nitrogen metabolism seen with added glutamine was attributed to the presence of small amounts of ammonia in glutamine solutions. Results using the derepressed mutant of *R. capsulata* indicate that glutamine itself may be the repressing signal (Table 1). In contrast, glutamine has been used as a derepressing nitrogen source for N_2-fixing bacteria (*e.g.* CLOSE and SHANMUGAM 1980).

Two alternative explanations for these apparently contradictory effects of glutamine are: 1) nitrogen regulation in diverse systems is materially different or 2) the glutamine may be physiologically unavailable to the sites of regulation in some culture conditions. The latter situation may arise any of several ways: 1) the small amounts of glutamine used in some experiments may be quickly depleted, 2) glutaminase-like activities may convert the glutamine to glutamate and ammonia with the ammonia being preferentially used, or 3) high levels of glutamine may shut down its own transport, thereby reducing its internal concentration. To determine the true conditions for repression, more precise experiments are needed comparing mutant and wild-type cells.

REGULATION OF ACTIVITY

It is tempting to extrapolate from the information concerning the regulation of synthesis of nitrogenase from the photosynthetic bacteria to the regulation of its activity via the activation-deactivation process (LUDDEN and BURRIS 1979). Indeed experiments by Zumft and Castillo (1978) showed a "switch-off" of nitrogenase activity *in vivo* by added ammonium salts, asparagine, or glutamine (the same compounds which repress synthesis) and a "switch-on" when these compounds were exhausted from the medium.

To elucidate the physiological role of nitrogenase inactivation, Nordlund and Eriksson (1979) compared *in vivo* nitrogenase activities from N-starved cultures with and without a brief ammonia pretreatment. Cultures without added ammonium salts had fully active nitrogenase. With ammonia pretreatment, an 80% decrease in activity was obtained which was almost completely restored by incubation with activating factor. Therefore, the "switching-off" of activity appeared to correlate with the addition of the adenine-like compounds to the Fe protein (LUDDEN and BURRIS 1979) and the "switch-on", with its removal by activating factor (NORDLUND and ERIKSSON 1979).

Is ammonia concentration or perhaps glutamine the signal for covalent modification and "switch-off"? Evidence that ammonia per se is not the signal for this phenomenon comes from the existence of the nitrogenase derepressed mutants, which have good activity in the presence of ammonia (WALL and GEST 1979). Additional experiments with these mutants are needed to determine the involvement of glutamine, if any.

Finally, it would seem prudent for a photosynthetic organism, growing photosynthetically in an anaerobic environment,

to be able to shut off energy demanding enzymes during darkness. This control was suggested by Ludden and Burris (1979) to explain why extracts contained deactivated enzyme when made from growing cells actively reducing N_2. They reasoned that harvesting is a dark process during which strict anaerobiosis is maintained and, consequently, energy is limited. This limitation signals the covalent modification of the Fe protein and stops reduction of substrates. Active *in vitro* preparations of the N_2-fixing complex were obtained from cultures starved for a nitrogen source and no longer growing (CARITHERS et al. 1979). Nitrogen limitation may override an energy limitation.

Evidence supporting a regulatory role for energy levels in nitrogen metabolism was reported. GS activity and synthesis appeared to be controlled via an energy signal in both *R. capsulata* (JOHANSSON and GEST 1977) and *E. coli* (TYLER 1978). Indeed the covalent modification of GS in *Klebsiella aerogenes* has been shown to change during the harvesting of cells (BENDER et al. 1977). This change was prevented by the pretreatment of cells with the detergent cetyltrimethyl ammonium bromide (BENDER et al. 1977). In this connection, it would be of interest to determine the effects of pretreatment with detergent on the activity of nitrogenase in extracts. In conclusion, there is reason to believe that the elucidation of nitrogen control of nitrogenase activity will be complicated by additional response to energy levels.

In summary, the photosynthetic bacteria offer interesting systems in which to study overlapping controls for synthesis and activity of the proteins required for nitrogen fixation. The identity of the molecular signals for repression/derepression and deactivation/activation will soon be elucidated. Ultimately, these investigations will determine whether these dual controls make the photosynthetic bacteria more energy-efficient diazotrophs.

ACKNOWLEDGMENTS

I would like to thank Dr. Howard Gest for introducing me to the wonders of the photosynthetic bacteria and Dr. David Emerich for critical discussions. This work was supported in part by the University of Missouri Institutional Biomedical Research Support Grant RR 07053 from N.I.H.

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