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The Regulation of Nitrogen Utilization in Enteric Bacteria

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OVERVIEW OF THE SYSTEM

It has long been known that enteric bacteria grow rapidly in a medium containing glucose as only source of carbon and ammonia as only source of nitrogen. They are, however, able to utilize many carbon compounds in place of glucose as sole source of carbon and energy and many organic and inorganic nitrogen compounds in place of ammonia as sole source of nitrogen. Generally the presence of glucose represses the enzymes required for the utilization of the alternative energy sources (catabolite repression), and ammonia similarly represses the enzymes required for the utilization of the alternative nitrogen sources (nitrogen regulation). In both instances the expression of the structural genes for these enzymes is positively regulated and the presence of glucose or ammonia prevents the initiation of transcription at the promoters for the respective genes or operons [Magasanik and Neidhardt, 1987].

To understand the physiology of nitrogen regulation it is necessary to realize that 85% of the cellular nitrogen is derived from the amino nitrogen of glutamate and 15% from the amide nitrogen of glutamine and to consider the biochemical mechanism of ammonia assimilation. Cells growing with an excess of ammonia synthesize glutamate by the reductive amination of α-ketoglutarate derived from the major source of carbon, a reaction catalyzed by the NADP-linked glutamate dehydrogenase; they use ammonia again to convert a portion of the glutamate to glutamine in a reaction catalyzed by glutamine synthetase (GS) that is coupled to the hydrolysis of ATP to ADP and Pi. In cells growing on limiting ammonia, derived from the degradation of organic nitrogen compounds or the reduction of inorganic nitrogen compounds such as nitrate or dinitrogen, the unfavorable equilibrium of the reaction catalyzed by glutamate dehydrogenase prevents the synthesis of glutamate by this pathway. In that case the cells synthesize glutamate by a two-stage process, first converting glutamate and ammonia to glutamine by means of GS, and then reacting glutamine with α-ketoglutarate and NADPH in a reaction catalyzed by glutamate synthase. This second reaction results in the synthesis of two molecules of glutamate, so that the overall effect of the two reaction steps is the synthesis of one molecule of glutamate from α-ketoglutarate, ammonia, and NADPH at the cost of the hydrolysis of ATP that provides the favorable equilibrium to the reaction sequence. It is immediately apparent that this pathway requires a greatly increased activity of GS, since in cells utilizing these reactions glutamine is an essential intermediate in the synthesis of all cellular nitrogen compounds. It makes therefore very good sense that the synthesis of GS is subject to nitrogen regulation and that the signal for nitrogen excess or deprivation is the intracellular concentration of glutamine [Reitzer and Magasanik, 1987].

The structural gene for GS, *glnA*, is a member of the complex *glnALG* operon. The other members of this operon, *glnG*(*ntrC*) and *glnL*(*ntrB*), are, respectively, the structural genes for nitrogen regulators I and II (NR_I and NR_{II}), responsible for the regulation of this and other operons in response to the availability of nitrogen. The transcription of the complex *glnALG* operon can be initiated at three promoters: *glnAp1* and *glnAp2*, located in this order upstream of *glnA*, and *glnLp*, located between *glnA* and the *glnLG*

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portion of the operon. The promoters *glnAp1* and *glnLp* have nucleotide sequences characteristic of the great majority of enterobacterial promoters and transcription is initiated at these promoters in cells grown with an excess of nitrogen by the common σ^{70}-RNA polymerase. Binding sites for NR_I overlap these promoters and consequently NR_I partially represses the synthesis of GS and its own synthesis in cells grown with an excess of nitrogen. As a result of this repression the intracellular concentration of NR_I in these cells is very low, amounting to approximately 5–10 molecules of NR_I-dimer per cell. This low intracellular concentration of NR_I is adequate for the activation of the initiation of transcription at *glnAp2* which is the immediate response to a shift of the cells to a medium deficient in nitrogen [Magasanik, 1988]. The *glnAp2* promoter does not resemble the majority of enterobacterial promoters, but has a nucleotide sequence first recognized as a common element located approximately 10 bp upstream from the transcriptional start sites of the operons composed of genes coding for the proteins responsible for dinitrogen fixation (*nif*) in *Klebsiella pneumoniae,* whose expression is activated by nitrogen deprivation; the position of this sequence, with the consensus CTGG-PyPuPyPu----TTGCA, suggested the possibility that the initiation of transcription at nitrogen-regulated promoters may require a different RNA polymerase [Ausubel, 1984]. In fact, it was soon shown by experiments using cell extracts that the product of a gene originally called *glnF* or *ntrA* but subsequently renamed *rpoN,* which was known to be essential for the expression of the *nif* genes and of *glnA* in response to nitrogen deprivation, was a new sigma-factor, σ^{54} [Hirschman et al., 1985; Hunt and Magasanik, 1985]. Experiments using purified components of *E. coli* showed furthermore that transcription of *glnA* was strongly stimulated by NR_I and NR_{II} together, but not at all by either one alone [Hunt and Magasanik, 1985]. Subsequent experiments revealed that NR_{II} brings about the phosphorylation of NR_I and that the actual activator of the initiation of transcription at *glnAp2* is NR_I-phosphate [Ninfa and Magasanik, 1986].

NR_I-phosphate exerts its effect on σ^{54}-RNA polymerase bound to the *glnAp2* promoter from its binding sites overlapping *glnAp1,* whose centers are located 108 and 140 bp upstream from the start of transcription. The high affinity of these sites and of the binding site overlapping *glnLp* ensures the exquisite sensitivity of the initiation of transcription of *glnAp2* to NR_I-phosphate: the repression exerted by NR_I at *glnLp* keeps NR_I at a very low intracellular concentration which nevertheless is adequate for activation of transcription at *glnAp2* when a deficiency of nitrogen causes NR_I to be phosphorylated [Reitzer and Magasanik, 1986].

Activation of transcription at *glnAp2* results in a rapid intracellular accumulation of GS, the product of *glnA,* and of NR_I, the product of *glnG.* The increased intracellular concentration of NR_I-phosphate enables the cell to activate the transcription of other nitrogen-regulated genes and operons, which, like the *glnALG* operon, are equipped with promoters depending on σ^{54}-RNA polymerase for the initiation of transcription, but with binding sites with less affinity for NR_I-phosphate than those of the *glnALG* operon; consequently, the immediate effect of nitrogen deprivation is the activation of transcription of the *glnALG* operon, which provides the increased level of GS required for the efficient assimilation of ammonia, followed by an increase in the level of different gene products to provide the cell with ammonia [Magasanik, 1988]. Among the systems that respond directly to an increase in the intracellular concentration of NR_I are the *glnHPQ* operon, with genes coding for components of a glutamine transport system [Claverie-Martin and Magasanik, 1991], *argT,* coding for the LAO (arginine, ornithine, and lysine) binding protein, and *hisQMP,* coding for components of the histidine-uptake system [Ames and Nikaido, 1985]. In addition, NR_I-phosphate activates the transcription of the *nifLA* operon and of the *nac* gene of *Klebsiella* which provide in turn the activators required for the nitrogen regulated formation of the proteins responsible for dinitrogen fixation and for the enzymes required for the degradation of histidine and proline, respectively: the product of *nifA* is the activator of transcription at the σ^{54}-RNA polymerase-dependent promoters of the other *nif* operons, while the product of *nac* is the activator of transcription of the σ^{70}-RNA polymerase dependent *hut* and *put* operons [Merrick, 1983; Bender, 1991].

The addition of ammonia to cells growing in a nitrogen deprived medium results in a rapid arrest of transcription initiation at *glnAp2* as a consequence of the dephosphorylation of NR_I-phosphate which is brought about by NR_{II} in combination with P_{II}, the product of the *glnB*

gene. In cells growing under nitrogen limitation, P_{II} is present at P_{II}-UMP which does not interfere with the phosphorylation of NR_I by NR_{II}. The enzyme responsible for the uridylylation of P_{II} is uridylyltransferase (UTase), the product of *glnD,* and this enzymatic activity is stimulated by α-ketoglutarate and inhibited by glutamine. The same enzyme is also responsible for the removal of the UMP from P_{II}-UMP, and this activity is stimulated by glutamine and inhibited by α-ketoglutarate. Therefore, the addition of ammonia to cells growing with nitrogen limitation which increases the intracellular concentration of glutamine and decreases that of α-ketoglutarate results in the conversion of P_{II}-UMP to P_{II} and consequently to the dephosphorylation of NR_I-phosphate and the arrest of transcription initiation at *glnAp2.* Conversely, deprivation of ammonia results in the uridylylation of P_{II} and consequently in the phosphorylation of NR_I and the activation of transcription at *glnAp2* [Reitzer and Magasanik, 1987].

It is apparent that because of these relationships, GS plays an important role in nitrogen regulation. It can be considered to be the intracellular sensor which responds to the extracellular stimulus, the presence of ammonia. In the absence of ammonia, NR_I-phosphate, directly and indirectly activates the transcription of the nitrogen-regulated operons. In the presence of ammonia, the sensor GS generates the intracellular signal, an increase in the concentration of glutamine, which is transmitted by the signal transducers UTase and P_{II} to NR_{II}, the modulator, which in turn inactivates the response regulator, NR_I-phosphate. Similarly, deprivation of ammonia makes it impossible for the sensor GS to maintain a sufficiently high intracellular concentration of glutamine, which in turn enables the modulator NR_{II} to activate the response regulator NR_I [Magasanik, 1988].

Not only the synthesis but also the activity of GS is regulated in response to the intracellular concentration of glutamine by P_{II} and UTase. P_{II} combines with the enzyme adenylyltransferase (ATase) to stimulate the adenylylation of GS that greatly reduces its activity. Conversely, P_{II}-UMP combines with ATase to bring about the deadenylylation of GS. The rapid inactivation of GS brought about by the adenylylation is of particular importance when cells grown under nitrogen deprivation are supplied with ammonia. The high level of GS in these cells results in the rapid production of glutamine, which, unless halted by the inactivation of GS, lowers the intracellular level of glutamate below that essential for protein synthesis [Kustu et al., 1984].

PHOSPHORYLATION AND DEPHOSPHORYLATION

The nucleotide sequence of the *glnL*(*ntrB*) and *glnG*(*ntrC*) genes of a *Bradyrhizobium* was determined by Nixon et al. [1986]. A comparison of these sequences with those of other regulatory genes enabled these authors to classify the products of these genes as paired members of two families: response regulators, sharing homology with the *glnG* product NR_I in the amino-terminal domains and modulators, sharing homology with the *glnL* product NR_{II} in the carboxy-terminal domains. By now, a large number of these paired regulators have been found in a large variety of bacteria, and it has been shown in a number of cases that the modulator brings about the phosphorylation of the response regulator [Stock et al., 1989; Albright et al., 1989].

Initially, NR_{II} reacts with ATP to bring about the transfer of the γ-phosphate group of ATP to the histidine in position 139 of NR_{II} [Weiss and Magasanik, 1988; Ninfa and Bennett, 1991]. This phosphate group is then transferred to an aspartate residue of NR_I, almost certainly to the one located in position 54. NR_I-phosphate is unstable due to autophosphatase activity; it has a half life of 3.5 min, compared to the half life of 5.5 h of the denatured product [Weiss and Magasanik, 1988].

The experiments with cell extracts clearly established the responsibility of NR_{II} for the phosphorylation of NR_I, but experiments with intact mutant cells with a deletion of *glnL* indicated the existence of an additional mechanism. These mutants had levels of GS comparable to those of the parent strain that were less influenced by the nitrogen source of the medium than those of the parent strain, and in such a mutant the initiation of transcription at *glnAp2* responded very sluggishly to changes in the availability of ammonia [Reitzer and Magasanik, 1985]. The nature of the alternative mechanism responsible for the phosphorylation of NR_I and of other response regulators has recently been discovered. The initial observations were that the response regulator CheY for bacterial mobility could catalyze its own phosphorylation by phosphoramidate or acetylphosphate, but not by ATP [Lukat et al., 1992], and that in intact cells lacking the modulators PhoR and CreC, acetylphosphate could bring about the phos-

phorylation of the response regulator for phosphate control, PhoB [Wanner and Wilmes-Riesenberg, 1992]. Subsequently, Feng et al. [1992] proved that in cells with a deletion of *glnL* the intracellular accumulation of acetylphosphate was responsible for the observed increase in the level of glutamine synthetase, that phosphoramidate, acetylphosphate, and carbamylphosphate were substrates for the autophosphorylation of NR_I, and that NR_I-phosphate generated in this manner was capable of activating the initiation of transcription at *glnAp2* in the absence of NR_{II}. Nevertheless, in cells with functional NR_{II}, growing in a medium with excess ammonia, the accumulation of acetylphosphate fails to bring about an increase in the level of glutamine synthetase. Apparently the phosphorylation of NR_I by acetylphosphate is too slow to overcome the rapid dephosphorylation of NR_I-phosphate by NR_{II}. Nitrogen regulation of gene expression therefore depends not so much on the ability of NR_{II} to phosphorylate NR_I using ATP as the phosphate donor, but primarily on the ability of NR_{II} to bring about in combination with P_{II} the dephosphorylation of NR_I-phosphate.

Although experiments using purified NR_{II} and P_{II} have shown that these proteins in the absence of ATP greatly increase the rate of dephosphorylation of NR_I-phosphate [Ninfa and Magasanik, 1986; Keener and Kustu, 1988], it is possible that still another protein is required for this reaction. As pointed out by Keener and Kustu [1988], the concentration of P_{II} required to bring about the dephosphorylation of NR_I-phosphate is 100 times greater than the concentration of P_{II} required to enable ATase to adenylylate GS. It is therefore possible that the P_{II} used in these experiments was contaminated by perhaps as little as 1% of another protein essential for the dephosphorylation of NR_I-phosphate. Nevertheless, the fact that deletion of only *glnB* results in high levels of GS in cells grown with an excess of ammonia clearly shows that P_{II} is essential for the dephosphorylation of NR_I-phosphate. We have some preliminary genetic evidence that the product of a gene linked to *glnB* plays a role in the dephosphorylation, but so far there is no biochemical evidence that the product of this gene enables NR_{II} and P_{II} to dephosphorylate NR_I-phosphate.

ACTIVATION OF TRANSCRIPTION

Following the discovery that transcription at *glnAp2* is not initiated by the most abundant RNA polymerase containing the σ^{70} subunit, but by a novel, minor polymerase with σ^{54} as its subunit, as many as 64 σ^{54}-dependent promoters have been identified in 22 different species of bacteria [Kustu et al., 1989]. The initiation of transcription at these promoters is not essential under all conditions of growth but rather serves to provide the cell with proteins required under special conditions, such as a deficiency in the source of nitrogen, the need to prevent excessive acidity due to the accumulation of formic acid, or the need to transport dicarboxylic acids into the cell as sources of energy.

The initiation of transcription by all types of RNA polymerase involves the binding of the polymerase to the promoter to form the closed RNA polymerase-promoter complex, followed by the melting of a stretch of DNA at the site of transcription initiation to form the open complex. In the case of σ^{70}-RNA polymerase the transition of the closed to the open complex can be accomplished without the help of another protein and does not require the hydrolysis of ATP, but in the case of σ^{54}-RNA polymerase this transition always requires an activator protein and the hydrolysis of ATP [Collado-Vides et al., 1991; Kustu et al., 1991].

The σ^{54}-dependent promoters differ in their affinity for RNA polymerase. Although the polymerase must contact the -GG-dinucleotide located approximately 24 bp upstream from the transcriptional start [Sasse-Dwight and Gralla, 1990], the affinity of the promoter for the polymerase is determined by nucleotides immediately upstream from the -GC- dinucleotide located approximately 12 bp from the transcriptional start site. Promoters with T, rather than C, in most of the four positions preceding the -GC- dinucleotide have high affinity for the polymerase; thus, the affinity of the *nifH* promoter which is very low is greatly improved by the change of its -CCCTGC- sequence to -TCTTGC- or -TTTTGC- [Cannon and Buck, 1992].

The activators responsible for the catalysis of the isomerization of the closed to the open complex bind to sites located more than 80 bp upstream from the transcriptional start site, a location too distant to allow direct contact between the activator and the polymerase-promoter complex [Kustu et al., 1989; Collado-Vides et al., 1991]. Moreover, the binding sites for NR_I can be moved more than 1,000 bp upstream or downstream from the *glnAp2* promoter and still permit NR_I-phosphate bound to these sites to activate transcription of the

glnALG operon [Reitzer and Magasanik, 1986]. These binding sites can therefore be defined as prokaryotic enhancers. It has been shown that the flexibility of the DNA makes it possible for the activator bound to a distant site to contact the polymerase at the promoter forming a DNA loop, and that the resulting increase in the local concentration of the activator is responsible for the success of the interaction [Su et al., 1990; Wedel et al., 1990].

All known activators of σ^{54}-dependent promoters share a highly homologous central domain, approximately 220 amino acids in length, that contains a binding site for ATP, and in addition, their carboxy-terminal domains contain a helix-turn-helix motif which enables these proteins to bind to specific sites on the DNA. On the other hand, only some activators, but not others, also share homology with NR_I in the aminoterminal domain that contains the aspartate residue which is the target of phosphorylation. Of particular interest with regard to nitrogen regulation is the fact that NifA, the activator of all *nif* operons with the exception of *nifLA,* closely resembles NR_I in the central domain, but not in the amino-terminal domain. The transcription of the *nifLA* operon is activated by NR_I-phosphate and results in the increased intracellular concentration of NifA. This unphosphorylated protein then activates the expression of the other *nif* operons; its activity is regulated in an as yet undiscovered manner by the product of *nifL* in response to oxygen and, perhaps, ammonia [Kustu et al., 1991].

The catalysis of the isomerization of the closed to the open σ^{54}-RNA polymerase-promoter complex by NR_I-phosphate requires ATP and presumably involves the contact of the central domain of NR_I-phosphate with the closed complex [Popham et al., 1989]. This view receives strong support from the observation that mutations that result in the alteration of the amino acid sequence in the central domain, and in particular those that alter the binding site for ATP, disable the ATPase activity of NR_I-phosphate as well as its ability to activate transcription [Weiss et al., 1991; Austin et al., 1991]. Furthermore, a mutation that changes a serine-residue in the central domain, located near the ATP-binding site, to phenylalanine enhances both the ATPase activity of NR_I-phosphate and its ability to activate transcription [Weglenski et al., 1989; Dixon et al., 1991].

The effectiveness of the activator depends on the occupation of the promoter by the polymerase. The affinity of *glnAp2* for σ^{54}-RNA polymerase is so great that in intact cells this promoter is always occupied by σ^{54} [Reitzer et al., 1987; Sasse-Dwight and Gralla, 1988]; this is not the case for a promoter with less affinity for the polymerase, such as *nifHp* [Morett and Buck, 1988]. Thus, in the case of *glnAp2,* but not in the case of *nifHp,* the activator bound to its distant site, will be able to make an effective contact with the closed polymerase-promoter complex whenever the flexibility of the DNA results in the proper alignment of the two partners. It is for this reason that maximal activation of transcription at a low affinity promoter, such as *nifHp*, depends on an additional protein, the integration host factor (IHF) [Hoover et al., 1990]. This protein binds to many specific sites on the chromosome of enteric bacteria and affects a variety of functions by bending the DNA by more than 140°. Binding sites for IHF are located between many σ^{54}-dependent promoters and the binding sites for their activators. Apparently, IHF exerts its effect by bending the DNA to hold the activator bound to its site in a position that allows it to make contact with the polymerase, whenever the polymerase binds to the promoter. Sydney Kustu and her co-workers [Hoover et al., 1990] have given a very plausible explanation for the preference of the cell for weak promoters with binding sites for IHF over strong promoters that do not depend on IHF. It is known that a mutation that increases the affinity of the *nifHp* promoter for RNA polymerase allows this normally NifA-specific promoter to be activated by NR_I, although no binding sites for this activator are located upstream of this promoter [Ow et al., 1985; Ray et al., 1990]: apparently, NR_I can bind non-specifically to DNA in the general vicinity of *nifHp,* and its chance to activate transcription at *nifHp* is greatly increased by high occupancy of this promoter by the RNA polymerase. This inappropriate activation is prevented by the low affinity of the normal *nifHp* promoter for the polymerase, and the specific activation by NifA bound to its site upstream of the promoter is assured by IHF binding to its site located between the NifA-binding site and the promoter.

In the case of *glnHp2*, another promoter with binding sites for IHF, it could be shown that in the absence of IHF, the flexibility of the DNA enables NR_I-phosphate to activate transcription irrespective of the side of the DNA helix to which it is bound. The presence of IHF, stimulates the activation of transcription when the

binding sites for NR_I and IHF are in their original positions, but strongly inhibits the activation of transcription when either its binding site or that for NR_I or both are moved to the opposite face of the helix [Claverie-Martin and Magasanik, 1991, 1992]. In the latter cases the bending of the DNA by IHF moves the NR_I-binding sites away from the RNA polymerase at the promoter. This is an interesting example of how a regulatory protein can positively or negatively affect transcription without direct contact with the components of the transcription system, although so far there has been no example of a σ^{54}-dependent promoter negatively regulated by IHF.

The critical event that renders NR_I capable of activating transcription is its conversion to NR_I-phosphate; but the fact that other activators such as NifA need not be phosphorylated to become effective clearly shows that the phosphate group of NR_I-phosphate does not have a direct role in the interaction of the central NR_I-domain with the polymerase-promoter complex. Five of the six known σ^{54}-promoters that are activated by NR_I-phosphate are associated each with at least two binding sites for NR_I located close to one another at the same face of the DNA helix [Weiss et al., 1992]; the only exception is *argP,* where no binding sites of NR_I have been detected [Ames and Nikaido, 1985]. In the case of *glnAp2,* there are two strong binding sites for NR_I with their centers located 108 and 140 bp upstream from the start of transcription initiation. NR_I and NR_I-phosphate bind equally well to a single one of these sites, but phosphorylation greatly increases the cooperative binding to two sites. A comparison of the ability of NR_I-phosphate to activate transcription at the *glnAp2* promoter on templates carrying two binding sites or a single binding site for NR_I showed that in the case of two binding sites, activation increased with the occupancy of the sites, but in the case of a single site, occupation of the site did not result in activation; rather the concentration of NR_I-phosphate had to be increased well above that required for the occupation of the single site to bring about the activation of transcription. These results indicate that the actual activator of transcription is not the dimeric NR_I-phosphate, but a tetramer resulting from the dimerization of the dimers. Apparently, a single NR_I-phosphate dimer bound to DNA interacts with a second dimer to form the critical tetramer [Weiss et al., 1992]. Other observations are in good accord with this view. The ATPase activity of NR_I-phosphate, which is essential for the activation of transcription, increases in a sigmoidal fashion with an increase in the concentration of NR_I-phosphate and this activity is stimulated by the addition of oligomeric DNA carrying two binding sites for NR_I [Weiss et al., 1991; Feng et al., 1992; Austin and Dixon, 1992].

We can therefore conclude that the role of phosphorylation is to stimulate dimerization of the NR_I-phosphate dimers. The interaction of the phosphorylated amino-terminal domains of NR_I, which is greatly facilitated by the binding of the dimers to neighboring sites on the DNA, apparently results in a conformational change in their central domains, creating the actual activator of transcription, the NR_I-phosphate tetramer.

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