Complex formation between AmtB and GlnK: an ancestral role in prokaryotic nitrogen control

A. Javelle and M. Merrick[1]
Department of Molecular Microbiology, John Innes Centre, Norwich NR4 7UH, U.K.

Abstract

Ammonium transport proteins belonging to the Amt family are ubiquitous in prokaryotes. In *Escherichia coli*, the AmtB protein and the associated P_{II} signal transduction protein (GlnK) have recently been recognized as an ammonium sensory system that effectively couples the intracellular nitrogen regulation (Ntr) system to external changes in ammonium availability. Given the almost invariant coupling of AmtB and GlnK in bacteria and archaea it seems probable that these two proteins may constitute an ancestral nitrogen-responsive system that has been coupled with a variety of unrelated nitrogen regulatory processes, which are now found in prokaryotes. The multiplicity of P_{II} proteins could therefore be considered to have evolved from an ancestral GlnK-like protein and to have subsequently been adapted to control many other aspects of nitrogen metabolism.

Introduction

Ammonium transporters

The ammonium transport (Amt) protein family comprises a unique and ubiquitous group of integral membrane proteins found in the three domains of life. This family comprises the Amt/Mep proteins, found in bacteria, archaea, fungi and plants, and the Rhesus proteins, which are found in animals from nematodes to man [1]. Nearly all prokaryotes encode at least one Amt protein and in some cases two or three homologues are present in a genome. The best studied of these proteins is presently *Escherichia coli* AmtB, which is a trimeric polytopic membrane protein in which each polypeptide has 11 transmembrane helices with an extra-cytoplasmic N-terminus and a cytoplasmic C-terminus [1–3].

P_{II} proteins

The prokaryotic *amt* genes are remarkable in one particular respect, namely that they are almost invariably found associated with a second gene (*glnK*) that encodes a small signal transduction protein [4]. GlnK is a member of the P_{II} protein family, a group of trimeric proteins that act as sensors of the cellular nitrogen status in prokaryotes and which have also been identified in plants [5]. GlnK forms a compact barrel with a relatively unstructured loop (the T-loop) protruding from the upper surface [6]. In cells that are subject to nitrogen starvation, Tyr-51 at the apex of the T-loop is covalently and reversibly modified. In *E. coli* and other proteobacteria this modification is uridylylation [7], whereas in *Streptomyces coelicolor* [8] and *Corynebacterium glutamicum* [9] it is adenylylation. In all these cases, reversible modification is mediated by the GlnD protein. However, the covalently modified tyrosine residue is not totally conserved in P_{II} proteins. Cyanobacterial P_{II} proteins are by contrast reversibly phosphorylated on a conserved serine (Ser-49) and in other organisms that have neither Tyr-51 nor Ser-49, e.g. *Bacillus subtilis*, there is no evidence for any form of modification [10].

The *glnKamtB* operon

The transcriptional linkage between *glnK* and *amtB* led us to propose that the GlnK and AmtB proteins might physically interact [4]. We recently confirmed that in *E. coli*, AmtB does form a membrane-bound complex with GlnK and that AmtB activity is regulated by the formation of this complex [11,12]. Complex formation is reversible and occurs within seconds in response to micromolar changes in the extracellular ammonium concentration [11]. GlnK has also been shown to bind to the membrane in an AmtB-dependent fashion in *Azotobacter vinelandii* [12], *B. subtilis* [10] and *C. glutamicum* [9]. These observations indicate that this interaction constitutes a novel signal-transduction pathway, which we believe could be ubiquitous in prokaryotes.

AmtB–GlnK complex formation as a component of the nitrogen regulation (Ntr) system in *E. coli*

The uridylylation state of GlnK governs its binding to AmtB [11] and this raises the question of whether ammonium binding to and/or transport through AmtB is necessary to propagate the signal that leads to uridylylation of GlnK. Using mutations that reduce or abolish AmtB activity, but not the amount and the targeting of the protein, we have shown that under physiological conditions, GlnK deuridylylation is completely dependent on AmtB activity. Hence in this situation ammonium enters the cell solely through the action of AmtB and an increase in the intracellular glutamine pool is intimately coupled with AmtB activity [11].

Key words: ammonium sensing, ammonium transport, nitrogen fixation, nitrogen regulation, P_{II} regulatory proteins, signal transduction.
Abbreviations used: Amt, ammonium transport; Ntr, nitrogen regulation.
[1]To whom correspondence should be addressed (email mike.merrick@bbsrc.ac.uk).

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These experiments confirm that in *E. coli* AmtB acts as a sensor of the extracellular ammonium concentration. Consequently, AmtB and GlnK can be considered as integral components of the nitrogen regulation (Ntr) system that until now has been characterized by just four proteins: uridylyltransferase (GlnD), GlnB (the other P_{II} protein in *E. coli*) and the two-component regulatory system NtrB/NtrC. In this case, NtrC is an activator of genes induced in nitrogen-limited conditions and control is effected by the balancing kinase/phosphatase properties of NtrB that are in turn determined by the interaction of NtrB with GlnB (see [5] for a review). The Ntr system has previously been considered only as a monitor of the intracellular nitrogen status but the incorporation of AmtB and GlnK rationally integrates sensing of the extracellular ammonium status into the system.

These results suggest that the control of AmtB activity is the primary cellular role of GlnK in *E. coli* and lend credence to the concept that this is the primary function of all GlnK proteins, which are encoded in a *glnKamtB* or *amtBglnK* operon. However, the role of AmtB as an ammonium sensor provides a mechanism whereby not only is AmtB activity regulated in response to the cellular demand for ammonium but the cytoplasmic pool of GlnK is also modulated rapidly in response to changes in the extracellular ammonium availability.

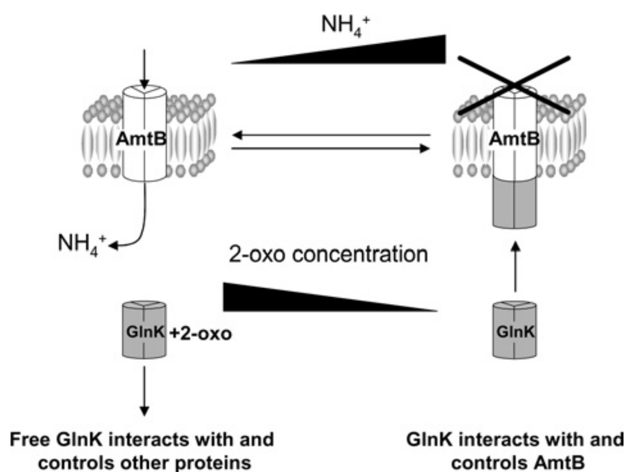
Nitrogen control in other bacteria

The Ntr system is found in the α-, β- and γ-proteobacteria but other major bacterial groups have quite different mechanisms of global nitrogen control. Amongst the actinobacteria, *C. glutamicum* is the most thoroughly analysed and its transcriptional regulation is by a repressor protein, AmtR, rather than by NtrBC homologues (see [13] for a review). AmtR activity is supposed to be controlled by interaction with GlnK, such that GlnK inactivates the repressor properties of AmtR but this has yet to be directly demonstrated [13]. It is therefore attractive to hypothesize that, as in *E. coli*, ammonium flux through AmtB leads to the sequestration of GlnK and thereby to the repression of AmtR-regulated genes.

B. subtilis is the best characterized of the firmibacteria and here the nitrogen control network involves three regulatory proteins, TnrA, GlnR and CodY (see [14] for a review). Under nitrogen-sufficient conditions, the major regulator TnrA binds to and inactivates glutamine synthetase but is consequently itself inactive as a transcriptional activator, e.g. of *amtBglnK* expression [15]. Mutations in *glnK* cause a reduction in *amtBglnK* expression [10], which would be consistent with a role for GlnK in fully activating TnrA, perhaps by promoting displacement of glutamine synthetase in nitrogen-limited conditions.

Finally, in cyanobacteria, global nitrogen control is mediated by an activator protein called NtcA, which is a member of the Crp activator family. In this case, the three *amt* genes are all not linked to the single P_{II}-encoding gene (*glnB*) and there have been no studies to date of potential sequestration of GlnB by any of the Amt proteins [16]. However, GlnB is required to activate NtcA-mediated gene expression and it may therefore interact directly with NtcA in a manner analogous to the potential TnrA–GlnK interaction in *B. subtilis* [17].

Figure 1 | Proposed model of the ancestral AmtB/GlnK system



Evolution of nitrogen control systems

The common theme of all these varied nitrogen control systems is the role played by P_{II} proteins in regulating, directly or indirectly, transcriptional activation or repression of a complex regulon. Given that many organisms encode multiple copies of the *glnKamtB* operon and can also encode a variety of P_{II} proteins [5], it is apparent that the complexity of these systems has evolved by gene duplication. In looking for the evolutionary origins of nitrogen control, by far the most highly conserved feature is the AmtB–GlnK pair and we would therefore propose that these two proteins constituted the ancestral ammonium-sensing system.

Such a system could have involved an ancestral Amt protein and a P_{II}-like protein that was responsive to intracellular changes caused by a flux of ammonium through the transporter. The common feature still found in most, if not all, P_{II} proteins is the fact that their interactions with other proteins can be modulated by the intracellular concentration of 2-oxoglutarate, which binds directly to the P_{II} molecule. Responsiveness to other metabolic signals, most notably the intracellular glutamine pool, is effected through covalent modification, which is not universal to P_{II} proteins. Hence it seems reasonable to suppose that an early AmtB/GlnK system would have sequestered GlnK to AmtB in response to excess flux of ammonium, which in turn would cause lowering of the 2-oxoglutarate pool. As the cell became more nitrogen-limited, the 2-oxoglutarate pool would rise causing GlnK to dissociate from AmtB. A consequence of this behaviour would be a rise in the intracellular pool of GlnK when the cell was nitrogen-limited and this would in turn allow GlnK (or its paralogues) to take on new roles in regulating the activities of transcriptional regulators or other nitrogen-metabolism-related enzymes (Figure 1).

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Conclusions

The widespread conservation of the AmtB–GlnK protein pair amongst prokaryotes suggests that the regulation of ammonium flux into cells through Amt proteins is strictly regulated in most organisms. The switch in the cellular location of GlnK that is a part of this process may well have been utilized in the evolution of a number of distinctly different nitrogen regulation systems. This paper has concentrated on the regulation of global gene expression in a variety of organisms but P_{II} proteins also control a number of enzymes, e.g. the recent recognition of the control, by GlnB, of a key enzyme in arginine biosynthesis in cyanobacteria [18] or the regulation of nitrogenase activity by the NifI proteins in the archaebacterium *Methanococcus maripaludis* [19]. Hence in an evolutionary sense, and probably still today, the AmtB protein appears to play a previously unrecognized critical role in facilitating the control of prokaryotic nitrogen metabolism in response to the availability of ammonium.

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Received 14 September 2004

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