Carbon-source-dependent nitrogen regulation in *Escherichia coli* is mediated through glutamine-dependent GlnB signalling

Mani Maheswaran and Karl Forchhammer

Institut für Mikrobiologie und Molekularbiologie der Justus-Liebig-Universität Giessen, Heinrich-Buff-Ring 26-32, D-35392 Giessen, Germany

Received 30 April 2003
Accepted 6 May 2003

The PII signal transduction proteins GlnB and GlnK are uridylylated/deuridylylated in response to the intracellular glutamine level, the primary signal of the cellular nitrogen status. Furthermore, GlnB was shown to be allosterically regulated by 2-oxoglutarate, and thus GlnB was suggested to integrate signals of the cellular carbon and nitrogen status. Receptors of GlnB signal transduction in *Escherichia coli* are the NtrB/NtrC two-component system and GlnE, an enzyme which adenylylates/deadenylylates glutamine synthetase. In this study, the authors investigated the effect of different carbon sources on the expression of the NtrC-dependent genes *glnA* and *glnK* and on the uridylylation status of GlnB and GlnK. With glutamine as nitrogen source, high levels of *glnA* and *glnK* expression were obtained when glucose was used as carbon source, but expression was strongly decreased when the cells were grown with poor carbon sources or when cAMP was present. This response correlated with the uridylylation status of GlnB, suggesting that the carbon/cAMP effect was mediated through GlnB uridylylation, a conclusion that was confirmed by mutants of the PII signalling pathway. When glutamine was replaced by low concentrations of ammonium as nitrogen source, neither *glnAglnK* expression nor GlnB uridylylation responded to the carbon source or to cAMP. Furthermore, glutamine synthetase could be rapidly adenylylated in vivo by the external addition of glutamine; however, this occurred only when cells were grown in the presence of cAMP, not in its absence. Together, these results suggest that poor carbon sources, through cAMP signalling, favour glutamine uptake. The cellular glutamine signal is then transduced by uridylyltransferase and GlnB to modulate NtrC-dependent gene expression.

**INTRODUCTION**

Assimilation of ammonia and acquisition of various nitrogen sources is tightly controlled in most bacteria. A prerequisite for efficient nitrogen control is the constant monitoring of the intracellular nitrogen status. Information on the cellular nitrogen status is integrated and transduced by a signalling protein termed PII. PII proteins constitute a highly conserved family of signal transduction proteins, which are found in all three domains of life (reviewed by Arcondeguy *et al.*, 2001). *Escherichia coli* possesses two closely related PII paralogues, GlnB and GlnK. GlnB is produced constitutively and it regulates the NtrB/NtrC two-component system (for a review, see Ninfa & Atkinson, 2000). NtrC is a key transcriptional activator of various nitrogen-controlled genes, among them *glnA*, encoding glutamine synthetase (GS) (reviewed by Magasanik, 1996), and *glnK*, encoding the second PII parologue (van Heeswijk *et al.*, 1996). Furthermore, GlnB controls the activity of GS by adenylylation/deadenylylation through a bifunctional enzyme (ATase), the product of the *glnE* gene (Shapiro & Stadtman 1968; Stadtman, 1990; Jaggi *et al.*, 1997). GlnK accumulates in nitrogen-limited cells to high levels, and contributes to the regulation of NtrC-dependent genes other than *glnA* under conditions of severe nitrogen limitation (Atkinson *et al.*, 2002b; Blauwkamp & Ninfa, 2002a). Cells lacking GlnK are highly impaired in surviving periods of nitrogen depletion (Blauwkamp & Ninfa, 2002b).

Both PII proteins are covalently modified by uridylylation at a conserved tyrosyl residue (Tyr-51) (Son & Rhee, 1987) that is located at the tip of a solvent-exposed loop (T-loop) (Jaggi *et al.*, 1996). Uridylylation and deuridylylation are catalysed by a bifunctional uridylyltransferase/uridylylremovase enzyme (UTase/UR), the *glnD* product, which responds to the cellular glutamine level (Adler *et al.*, 1975). In the presence of high intracellular glutamine levels, signalling

**Abbreviations:** GS, glutamine synthetase; UTase/UR, uridylyltransferase/uridylylremovase.
nitrogen-excess conditions, uridylyremovase activity of GlnD dominates, causing demodification of uridylylated PII proteins. Low glutamine levels, signalling nitrogen deficiency, promote the uridylytransferase activity, leading to uridylylation of PII (Jiang et al., 1998a).

The interaction of the PII proteins with signal receptors is modulated by uridylylation of the T-loop. One receptor of PII signalling is the adenylytransferase/removase enzyme GlnE (ATase), which regulates the activity of GS by reversible adenylylation. The default mode of ATase, which is stimulated by glutamine and by non-uridylylated PII, is adenylylation of GS. Upon uridylylation of PII, a different complex is formed, which switches the activity of ATase towards deadenylylation and thereby activation of GS (Rhee et al., 1985; Jiang et al., 1998c; Jaggi et al., 1997). Non-modified GlnB forms a complex with NtrB, which dephosphorylates the response regulator NtrC, thus switching off transcription of glnA (Jiang & Ninfa, 1999; Pioszak et al., 2000) and of other NtrC-dependent genes like glnK (Atkinson et al., 2002a). By contrast, GlnB-UMP is not able to form a complex with NtrB. In this state, the histidine kinase activity of NtrB dominates, and by phosphoryl transfer, NtrC is phosphorylated and is now transcriptionally active (Jiang & Ninfa, 1999). In addition to uridylylation, GlnB is regulated allosterically by the effector molecule 2-oxoglutarate as shown by Ninfa and colleagues in a series of in vitro analyses (Jiang et al., 1998a, b, c). Of the three binding sites for 2-oxoglutarate within the trimeric GlnB protein, only one site is occupied with high affinity, whereas affinity of the second and third sites is decreased by negative cooperativity. Only at high 2-oxoglutarate concentrations does complete occupation of GlnB with this effector molecule occur. This state impairs complex formation of non-uridylylated GlnB with NtrB, which finally results in phosphorylation and, thus, activation of NtrC (Jiang & Ninfa, 1999). From these biochemical properties, it was proposed that GlnB integrates signals of the nitrogen status (by uridylylation) with a signal of central carbon metabolism (2-oxoglutarate) to co-ordinate the expression of key enzymes of nitrogen assimilation in response to the nitrogen and carbon state. However, transduction of carbon signals by PII proteins in E. coli has not yet been demonstrated in vivo.

Another link between carbon and nitrogen regulation has been demonstrated for the regulation of expression of the glnALG operon. Its transcription is initiated from tandem promoters (glnAp1 and glnAp2), which are under carbon and nitrogen control (reviewed by Reitzer, 1996, and by Magasanik, 1996). The activity of the weak, $\sigma^{70}$-dependent glnAp1 promoter requires binding of the CRP–cAMP complex to an upstream activating sequence. The CRP–cAMP complex is formed when cells are grown with a carbon source that is not transported by the phospho- transferase system (PTS). The utilization of PTS sugars like glucose leads to a decrease in cellular cAMP levels and dissociation of the complex, thereby causing catabolite repression of a large number of genes (reviewed by Saier et al., 1996). Located 116 bp downstream of glnAp1 is a strong $\sigma^{54}$-dependent promoter, glnAp2, which is activated by NtrC-phosphate. Concomitant with the activation of glnAp2, NtrC-phosphate blocks transcription from the weak glnAp1 promoter. Thus, when NtrC-phosphate accumulates during nitrogen-limited growth, the glnALG operon is almost exclusively transcribed to high levels from glnAp2 (Magasanik, 1996). A recent investigation showed that transcription from glnAp2 also responds to catabolite repression. Elevated levels of cAMP, through formation of the CRP–cAMP complex, caused a 21-fold downregulation of transcription from glnAp2 (Tian et al., 2001). However, the mechanism by which the CRP–cAMP complex affects transcription from glnAp2 remains unclear.

To elucidate the mechanism whereby carbon signals affect NtrC-dependent gene expression, we studied the in vivo response of the PII signalling system to different carbon sources. Expression of the NtrC-dependent genes glnA and glnK was monitored and compared to the uridylylation status of the PII proteins. All responses of glnA and glnK expression to carbon or cAMP signals were in accord with alterations in the uridylylation status of the PII proteins. We conclude that the effect of carbon source on NtrC-dependent gene expression is mediated through cAMP-dependent glutamine uptake, thereby affecting the glutamine-sensitive ATase–PII signalling system.

**METHODS**

**Bacterial strains and growth conditions.** The bacterial strains used in this work are shown in Table 1. The minimal medium used in this study was based on M9 minimal medium (Sambrook et al., 1989) with the following modifications. The medium was supplemented

---

**Table 1. Strains of E. coli used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>YMC10</td>
<td>endA1 thi-1 hsdR17 supE44 ΔlacU169</td>
<td>Backman et al. (1981)</td>
</tr>
<tr>
<td>RB9060</td>
<td>As YMC10, ΔglnB</td>
<td>Bueno et al. (1985)</td>
</tr>
<tr>
<td>HS10</td>
<td>As YMC10, ΔglnK</td>
<td>Strobel (1998)</td>
</tr>
<tr>
<td>YMC26</td>
<td>As YMC10, glnD::Tn10</td>
<td>Reitzer &amp; Magasanik (1985)</td>
</tr>
</tbody>
</table>
Carbon-source-dependent nitrogen regulation

RESULTS AND DISCUSSION

The expression of nitrogen-regulated genes is affected by the carbon source

To investigate the effect of different carbon sources on the P_{II} signal transduction system in E. coli, wild-type cells were grown in minimal medium supplemented with various carbon sources using glutamine as nitrogen source. Minimal medium with glutamine as sole nitrogen source is commonly referred to as a ‘nitrogen-limiting’ condition, and was shown to lead to the activation of NtrC-dependent gene expression (Bueno et al., 1985; Atkinson & Ninfa, 1998). Cells from the mid-exponential phase of growth were harvested and transferase activity of GS (GSt) was determined as a measure of GS quantity, and thus of glnA expression (Table 2). Furthermore, protein extracts were analysed by immunoblotting of non-denaturing gels to determine the abundance and modification state of the PII proteins GlnB and GlnK, using GlnB- or GlnK-specific antibodies (Fig. 1). A high level of GS, activity was measured in glucose + glutamine-grown cultures, as expected. Xylose as carbon source also supported high GS, activities. However, in the presence of the other carbon sources, GS levels were significantly lower. With glycerol, galactose or succinate as carbon source, GS activities were even lower than under conditions of excess ammonium supply (glucose with 37 mM ammonium chloride). Immunoblot analysis of non-denaturing gels using GlnK-specific antibodies showed that GlnK was undetectable in cells that were grown with those carbon sources, which also repressed glnA expression (Fig. 1B). Only in glucose- or xylose-grown cells was GlnK produced in detectable amounts. Under those conditions, GlnK was almost completely present in the fully uridylylated state, as revealed by comparing the mobility of GlnK to that of cells exposed to ammonium in order to deuridylylate GlnK (Fig. 1B, lane C). As expected, GlnB, whose synthesis is not subject

Table 2. Effect of different carbon sources on the expression of GS in E. coli YMC10 compared to the expression in ammonium-excess conditions

<table>
<thead>
<tr>
<th>Growth conditions</th>
<th>GS, specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose + glutamine</td>
<td>2490 ± 306</td>
</tr>
<tr>
<td>Glycerol + glutamine</td>
<td>295 ± 106</td>
</tr>
<tr>
<td>Galactose + glutamine</td>
<td>220 ± 74</td>
</tr>
<tr>
<td>Succinate + glutamine</td>
<td>203 ± 43</td>
</tr>
<tr>
<td>Xylose + glutamine</td>
<td>1430 ± 221</td>
</tr>
<tr>
<td>Glucose + 0.2 % NH₄Cl</td>
<td>411 ± 19</td>
</tr>
</tbody>
</table>

Glutamine synthetase (GS) assays. Frozen cells were thawed and resuspended in wash solution containing 1 % (w/v) KCl and 0.1 mg ml⁻¹ CTAB (N-cetyl-N,N,N-trimethylammonium bromide) and were incubated in this buffer for 3 min on ice to permeabilize the membranes. Then the cells were centrifuged and resuspended in reaction buffer without Mg²⁺ ions, and the γ-glutamyl transferase activity of glutamine synthetase (GS) activity was determined as described by Pahel et al. (1982). GS, specific activity is given in nmol γ-glutamylhydroxamate formed min⁻¹ (mg protein)⁻¹. The adenylylation status of GS was estimated by measuring the GS, activity in the presence or absence of 67 mM MgCl₂ (Stadtman et al., 1979). The cell suspension in magnesium-free reaction mixture was divided into two parts, one of which was supplemented with 67 mM MgCl₂. GS, activity was determined in the two parallel samples. The mean number of adenylylated subunits of GS was calculated from the quotient (Q) of GS, activity in the presence or absence of MgCl₂ in the assay, according to the following equation: n (number of adenylylated subunits of GS) = (1 - Q) x 12.

Determination of the modification status of GlnB and GlnK.

The modification status of the P_{II} proteins GlnB and GlnK was determined by non-denaturing polyacrylamide gel electrophoresis (Forchhammer et al., 1999) followed by immunodetection of the P_{II} proteins using appropriate antibodies. The frozen cells (see above) were thawed and lysed in a buffer containing 50 mM Tris/HCl pH 7.4, 4 mM EDTA, 1 mM DTT, 0.5 mM benzamidine and 0.5 mM PMSF by grinding the cells with glass beads (0.11 mm; Sigma-Aldrich) in a Hybaid Ribolysor (Hybird). Cell debris and glass beads were removed by centrifugation and the protein concentration in the supernatant was estimated according to the method of Bradford (1976), as modified by Rippka (1988), and instead of 3rd mM NH₄Cl, either 14 mM glutamine or 2 mM NH₄Cl was used as a poor nitrogen source, as indicated. The carbon sources (glucose, glycerol, xylose, galactose or succinate) were used at 0.4 % (w/v) final concentration. Cells were grown routinely at 37 °C under aerobic conditions. The growth of the cells was measured spectrophotometrically by following the optical density of the culture (OD₆₀₀). The cultures that were used for measurements were initiated by diluting a cell suspension from an overnight-grown culture in the appropriate medium to an initial OD₆₀₀ of 0–0.05. When the day-culture reached an OD₆₀₀ between 0.40 and 0.45, 2 ml aliquots were removed to reaction tubes and the tubes were rapidly chilled in liquid nitrogen for 5 s. The chilled, but not frozen, cell suspension was immediately centrifuged at 4 °C and the cell pellet was quickly frozen in liquid nitrogen and stored at −70 °C until its use for various assays.

Effect of different carbon sources on the expression of GS in E. coli YMC10 compared to the expression in ammonium-excess conditions

Cells were grown in minimal salt medium as described in Methods, and GS, activity [nmol γ-glutamylhydroxamate formed min⁻¹ (mg protein)⁻¹] was determined from cells in the mid-exponential phase of growth. Mean values and standard deviations from three independent experiments are shown.
to nitrogen control (Liu & Magasanik, 1993), could be detected under all growth conditions. The uridylylation status of GlnB correlated with the levels of GS activity and GlnK accumulation: in the presence of glucose or xylose, GlnB was highly uridylylated, whereas in the presence of glycerol, galactose or succinate its degree of uridylylation was much lower (Fig. 1A).

The carbon source effect on NtrC-dependent gene expression is mediated by cAMP and by the GlnB signal transduction system

The above preliminary experiment suggested that the PII signal transduction system perceives a signal from the carbon source of the cells and thereby affects expression of the NtrC-dependent genes glnA and glnK. Recently, down-regulation of glnA expression by the CRP–cAMP complex was reported in E. coli (Tian et al., 2001); however, the mechanism of this effect remained elusive. To investigate whether the effect of carbon source observed on the PII signal transduction system was related to the cAMP effect reported by Tian et al. (2001), we studied the expression of glnA in various mutants of the PII signalling system, in the presence and absence of 2 mM cAMP. Cells of the wild-type and of glnB, glnD and glnK mutants were grown in minimal medium with glutamine as sole nitrogen source and with either glucose or glycerol as carbon source, thus leading to high or low levels of NtrC activation as deduced from the previous experiment. As above, GS activity was determined (Table 3) and the quantity and modification status of the GlnB and GlnK proteins were analysed by immunoblotting (Fig. 2). Consistent with the report by Tian et al. (2001), addition of cAMP to glucose+glutamine-grown cells led to a strong decrease of GS activity, reaching a level that was similar to that of glycerol-supplemented cells. No further reduction of GS levels was obtained when cAMP was added to glycerol-supplemented cultures. These results are consistent with the report by Tian et al. (2001), which showed that the carbon effect on glnA expression is mediated through the signalling molecule of the carbon source, cAMP. These authors suggested that the CRP–cAMP complex negatively affects transcription from the NtrC-dependent glnAp2 promoter. As shown in Table 3, no carbon effect on GS levels could be detected in a GlnB-deficient mutant and only a slight reduction was determined from cAMP-treated cells. In contrast to GlnB, the GlnK protein seems to play no major role in the transmission of carbon/cAMP signals: in a GlnK-deficient mutant, low GS levels were obtained in glycerol-grown cells as well as in cAMP-supplemented cultures, as in the wild-type. In a GlnD-deficient mutant, which is unable to uridylylate the PII proteins, the GS levels remained low, as compared to the other strains.

Table 3. Effect of cAMP and glycerol on the expression of GS in E. coli wild-type and glnB, glnD and glnK mutants

<table>
<thead>
<tr>
<th>Growth conditions (0-2 %, w/v, glutamine as N source)</th>
<th>GS, specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>YMC10 (WT)</td>
</tr>
<tr>
<td>Glucose</td>
<td>2490 ± 306</td>
</tr>
<tr>
<td>Glucose + cAMP</td>
<td>347 ± 54</td>
</tr>
<tr>
<td>Glycerol</td>
<td>295 ± 106</td>
</tr>
<tr>
<td>Glycerol + cAMP</td>
<td>184 ± 99</td>
</tr>
</tbody>
</table>
The uridylylation status of GlnB (Fig. 2A) correlated with GS activity, cAMP and glycerol caused deuridylylation of GlnB in the wild-type and in the GlnK-deficient strain HS10. Whether the residual uridylylation of GlnB in HS10 as compared to wild-type is of significance remains to be elucidated. As expected, GlnB was not uridylylated in the glnD mutant. Together, these results confirmed the preliminary conclusion that the carbon effect on glnA expression is mediated through GlnB uridylylation.

Accumulation of the GlnK protein (Fig. 2B) showed a similar pattern as observed with GS activity in wild-type cells: glycerol as carbon source or the presence of cAMP repressed GlnK accumulation. In contrast to the constitutive high GS activities in the glnB mutant, synthesis of GlnK was still repressed by cAMP or glycerol in this mutant. This might suggest that the GlnK protein, in the absence of GlnB, is sufficient to regulate its own synthesis although it is unable to regulate glnA expression properly. This conclusion is in accord with recent studies by Atkinson et al. (2002a), where they showed that the expression of glnk requires higher levels of activated NtrC than that of glnA. Therefore, a partial inhibition of NtrC activity by GlnK might be sufficient to decrease glnk expression although it might be insufficient to affect glnA expression. As expected, glnK was not expressed in the glnD mutant, in which GlnB is permanently unmodified.

The carbon/cAMP effect on GlnB signal transduction depends on extracellular glutamine

The above experiment showed that uridylylation of the GlnB signalling protein responds to the carbon source or to the cAMP levels and it strongly suggested that the GlnB signalling protein is required to transmit the carbon/cAMP signal towards NtrC-dependent gene expression. These conclusions imply that the signal of the carbon source is perceived through the GlnD enzyme (UTase/UR), which is known to respond to the cellular glutamine levels. Therefore, we speculated whether the observed carbon/cAMP effects might result from different permeability of the cells to the exogenous nitrogen source glutamine when growing with different carbon sources or in the presence of cAMP. To examine this possibility, the nitrogen source glutamine was replaced by limiting amounts of ammonium, which were adjusted such that they caused nitrogen-poor conditions. Preliminary experiments revealed that addition of 2 mM ammonium chloride led to derepression of glnA expression in glucose-supplemented minimal medium when cells were harvested at the mid-exponential phase of growth (OD600 approx. 0.4), which is also in accord with the results from Atkinson et al. (2002a). Under those conditions, using either glucose or glycerol as carbon source, the four strains (wild-type, glnB, glnD and glnK mutants) were grown in the presence or absence of 2 mM cAMP. From cells.
harvested at the mid-exponential phase of growth, GS$_t$ activity was determined (Table 4) and the GlnB and GlnK proteins were analysed as above (Fig. 3). Clearly, GS$_t$ activity in wild-type cells was not decreased by the addition of cAMP and was only slightly affected by the presence of glycerol. Similarly, the $glnK$ mutant, which displayed a strong response to cAMP and to the carbon source in the presence of glutamine, no longer responded to the addition of cAMP. Its GS$_t$ activity in the presence of glucose was twofold elevated compared to the wild-type and was similar to the wild-type in the presence of glycerol, regardless of the presence of exogenous cAMP. The $glnB$ mutant again exhibited constitutively high GS$_t$ activity and the $glnD$ mutant showed low levels of GS$_t$ activity.

In the presence of 2 mM ammonium, synthesis of GlnK, like that of GS, no longer responded to the carbon source or towards cAMP (Fig. 3B). The GlnK protein that was detected by immunoblotting was present in its completely uridylylated state. The uridylylation status of the GlnB protein again paralleled the expression level of the NtrC-dependent genes $glnA$ and $glnK$: a high degree of GlnB uridylylation was detected in the wild-type and in the $glnK$ mutant, regardless of the carbon source or of cAMP (Fig. 3A). As expected, no GlnB modification occurred in the $glnD$ mutant. The result of this experiment showed unequivocally that, when glutamine was replaced by a limiting amount of ammonium, GlnB signal transduction and NtrC-dependent gene expression did not respond to the

Table 4. Effect of cAMP and glycerol on the expression of GS in E. coli wild-type and $glnB$, $glnD$ and $glnK$ mutants in the presence of limiting amounts of ammonium as nitrogen source

<table>
<thead>
<tr>
<th>Growth conditions (2 mM ammonium as N source)</th>
<th>GS$_t$ specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>YMC10 (WT)</td>
</tr>
<tr>
<td>Glucose</td>
<td>1201 ± 114</td>
</tr>
<tr>
<td>Glucose + cAMP</td>
<td>1281 ± 171</td>
</tr>
<tr>
<td>Glycerol</td>
<td>856 ± 399</td>
</tr>
<tr>
<td>Glycerol + cAMP</td>
<td>1108 ± 13</td>
</tr>
</tbody>
</table>

Fig. 3. Effect of glucose, glycerol and cAMP on the uridylylation status of GlnB and GlnK in E. coli wild-type cells and various mutants of the P II signalling system in the presence of limiting amounts of ammonium. E. coli wild-type (WT), glnD (D), glnB (B) and glnK (K) mutant cells were grown in a medium containing 2 mM ammonium chloride as sole nitrogen source and glucose or glycerol as carbon source, in the presence or absence of 2 mM cAMP, as indicated. The uridylylation status was analysed as described in Fig. 1. (A) Immunodetection with GlnB-specific antibodies. (B) Immunodetection with GlnK-specific antibodies. For the deuridylylation control, (lane C), an extract was prepared as described in Fig. 1(B), except that the cells were treated for 5 min with 0.2% ammonium chloride.
carbon source or to cAMP. Therefore, poor carbon sources or cAMP are not directly sensed by the PII signalling system. More plausibly, when glutamine is used as nitrogen source, the cells might experience nitrogen-sufficient conditions in the presence of elevated cAMP levels.

**In the presence of cAMP, extracellular glutamine elicits a nitrogen-excess signal**

Next, an experiment was performed to test whether exogenously added glutamine directly elicits a nitrogen-excess signal in the presence of cAMP. The cellular nitrogen status was monitored through determination of the adenylylation status of GS. Adenylylation of GS responds to the intracellular nitrogen status, determined by the cellular glutamine level, in a dual way: adenylylation is stimulated by non-uridylylated PII (which accumulates in the presence of elevated glutamine levels) and also responds directly to elevated glutamine levels (Jaggi et al., 1997; Jiang et al., 1998c). Therefore, increased cellular glutamine pools are reflected by increased GS adenylylation. Cells of wild-type and of the *glnB* and *glnK* mutants were grown in ammonium-limited, glucose-supplemented medium, in the absence or presence of 2 mM cAMP. At the mid-exponential phase of growth, glutamine was added to the cultures (14 mM final concentration), and over a 20 min time period, samples were removed and the adenylylation state of GS was assayed. As shown in Fig. 4(A), in wild-type cells, the adenylylation state of GS rapidly and permanently increased upon the addition of glutamine, but only when the cells had been grown in the presence of cAMP. By contrast, in the absence of cAMP, addition of glutamine caused only a slight and transient increase in GS adenylylation. In the GlnK-deficient strain, a similar cAMP-dependent GS adenylylation was observed (Fig. 4C). In the *glnB* mutant, GS was already highly adenylylated at the beginning of the experiment (Fig. 4B), since GlnB is required for the deadenylylation of GS under nitrogen-limited conditions (Jiang et al., 1998c; Forchhammer et al., 1999). Thus, in the absence of GlnB, GS is highly adenylylated even under nitrogen-poor conditions, and no further increase can be observed upon the addition of glutamine.

Another way to monitor an increase in intracellular glutamine levels is to analyse glutamine-dependent deuridylylation of GlnB. Wild-type cells were grown in glucose-supplemented, ammonium-limited medium in the presence or absence of 2 mM cAMP. Following the addition of 14 mM glutamine to the cultures, aliquots were removed after 0, 4, 10 and 20 min and the uridylylation status of GlnB was revealed by non-denaturing PAGE and immunoblotting. As shown in Fig. 5, GlnB is almost completely uridylylated prior to the addition of glutamine (*t*0). In the absence of cAMP only a minor and transient deuridylylation of GlnB can be observed upon the addition of glutamine, and the protein returns to its highly uridylylated state. By contrast, in the presence of cAMP, the addition of glutamine causes a drastic and permanent deuridylylation of GlnB. This result shows that deuridylylation of GlnB is a direct consequence of glutamine addition to cAMP-treated cells and corroborates our suggestion that cAMP increases the cellular glutamine level when glutamine is used as nitrogen source.

To reveal the concentration of external glutamine required to cause the observed effects, wild-type cells were grown as above and different amounts of glutamine, ranging from 1·4 mM to 21 mM were added at the mid-exponential phase of growth. After different times, aliquots were removed and the adenylylation state of GS was analysed. The result, shown in Fig. 6, reveals that at least 5 mM glutamine is required to elicit a measurable response. The effect saturates at concentrations above 14 mM, indicating...
the involvement of a mechanism with low affinity for glutamine.

L-Glutamine transport in *E. coli* has been shown to occur through a well-characterized high-affinity-binding-protein-dependent system and poorly characterized low affinity system(s). The high-affinity system, encoded by the *glnHPQ* operon, is under the control of NtrC (Willis et al., 1975; Claverie-Martin & Magasanik, 1991), and is therefore activated when the cells grow with glucose as carbon and glutamine as nitrogen source. Despite the presence of glutamine at concentrations as high as 14 mM, GlnB signals nitrogen deficiency. It follows, therefore, that the uptake rate limits glutamine utilization, since this concentration exceeds the *Kₘ* of the *glnHPQ* transporter for glutamine (*Kₘ* = 0.1–0.2 μM) by several orders of magnitude (McFall & Newman, 1996). However, when the cells are cAMP-treated or grown with poor carbon sources, high concentrations of external glutamine are perceived as a nitrogen-excess condition, indicating an accelerated glutamine transport into the cells by low-affinity system(s). One of these could involve the osmotic-shock-sensitive L-glutamate-L-asparate transport system (Weiner & Heppel, 1971). Its binding protein has a low affinity for glutamine and synthesis of this protein was shown to be repressed in growth on glucose as opposed to succinate (Willis & Furlong, 1975), suggesting that synthesis of this transport system is under catabolite repression. A further way by which the external glutamine could increase the intracellular glutamine status involves periplasmic glutaminase activity, uptake of hydrolysed ammonium and re-assimilation of ammonium by intracellular GS activity. *E. coli* possesses a periplasmic asparaginase enzyme, the *asnB* product L-asparaginase II, which displays low glutaminase activity and whose expression requires the CRP–cAMP complex (McFall & Newman, 1996). Elucidation of the mechanistic details by which glutamine permeates CAMP-treated cells requires further investigation.

**Outlook**

Analysing the carbon effect on the PII-signalling system and NtrC-dependent gene expression in the presence of glutamine as nitrogen source revealed an indirect effect of the carbon source on nitrogen regulation. This occurs by catabolite repression influencing glutamine uptake. If we wish to address the question concerning a direct integration of carbon and nitrogen signals by the PII protein, *in vivo* conditions have to be chosen that avoid the use of glutamine as nitrogen source. Limiting amounts of ammonium seem to be appropriate for this purpose. Under such conditions, however, *glnA* and *glnK* expression did not respond significantly to the nature of the carbon source, as shown in this study. Therefore, it remains to be demonstrated whether GlnB, in addition to signalling the nitrogen status through a well-characterized high-affinity-binding-protein (PII) system, may also influence carbon-distribution patterns. The PII system is known to function as a carbon catabolite activator (Claverie-Martin & Magasanik, 1991), and is therefore involved in the integration of both nitrogen and carbon signals. The involvement of a mechanism with low affinity for glutamine is therefore consistent with a role of GlnB as a sensor of nitrogen status. Understanding the mechanism by which cAMP influences GlnB activity is, however, a prerequisite for a complete understanding of the role of GlnB in nitrogen regulation.

**ACKNOWLEDGEMENTS**

We thank Heike Strobel for providing the GlnK-deficient strain HS10 and Mike Merrick for stimulating discussions. M. M. is a fellow of the
International Quality Network Program (IQN) sponsored by DAAD at the University of Giessen. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (Fo 195/4-1).

REFERENCES


http://mic.sgmjournals.org


