Molecular Microbiology (1999) **32**(2), 301–313

Characterization of the GlnK protein of *Escherichia coli*

Mariette R. Atkinson and Alexander J. Ninfa*
Department of Biological Chemistry, University of Michigan Medical School, Ann Arbor, MI 48109-0606, USA.

Summary

The GlnK and PII signal transduction proteins are paralogues that play distinct roles in nitrogen regulation. Although cells lacking GlnK appear to have normal nitrogen regulation, in the absence of PII, the GlnK protein controls nitrogen assimilation by regulating the activities of the PII receptors glutamine synthetase adenylyltransferase (ATase) and the kinase/phosphatase nitrogen regulator II (NRII or NtrB), which controls transcription from nitrogen-regulated promoters. Here, the wild-type GlnK protein and two mutant forms of GlnK were purified, and their activities were compared with those of PII using purified components. GlnK and PII were observed to have unique properties. Both PII and GlnK were potent activators of the phosphatase activity of NRII, although PII was slightly more active. In contrast, PII was approximately 40-fold more potent than GlnK in the activation of the adenylylation of glutamine synthetase by ATase. While both GlnK and PII were readily uridylylated by the uridylyltransferase activity of the signal-transducing uridylyltransferase/uridylyl-removing enzyme (UTase/UR), only PII∼UMP was effectively deuridylylated by the UR activity of the UTase/UR. Finally, there were subtle differences in the regulation of GlnK activity by the small molecule effector 2-ketoglutarate compared with the regulation of PII activity by this effector. Altogether, these results suggest that GlnK is unlikely to play a significant role in the regulation of ATase in wild-type cells, and that the main role of GlnK may be to contribute to the regulation of NRII and perhaps additional, unknown receptors in nitrogen-starved cells. Also, the slow deuridylylation of GlnK∼UMP by the UTase/UR suggests that rapid interconversion of GlnK between uridylylated and unmodified forms is not necessary for GlnK function. One mutant form of GlnK, containing the alteration R47W, was observed to lack specifically the ability to activate the NRII phosphatase *in vitro*; it was able to be uridylylated by the UTase/UR and to activate the adenylylation activity of ATase. Another mutant form of GlnK, containing the Y51N alteration at the site of uridylylation, was not uridylylated by the UTase/UR and was defective in the activation of both the NRII phosphatase activity and the ATase adenylylation activity.

Received 28 October, 1998; revised 15 January, 1999; accepted 19 January, 1999. *For correspondence. E-mail aninfa@umich.edu; Tel. (+1) 734 763 8065; Fax (+1) 734 763 4581.

Introduction

The PII signal transduction protein of *Escherichia coli*, product of *glnB*, plays a key role in the regulation of nitrogen assimilation in response to intracellular signals of nitrogen and carbon status. PII activates the phosphatase activity of the *glnL* (*ntrB*) product, NRII (NtrB) and, by so doing, controls the extent of phosphorylation and activity of the enhancer-binding transcription factor NRI (NtrC) encoded by *glnG* (*ntrC*) (Ninfa and Magasanik, 1986; Keener and Kustu, 1988; Kamberov *et al*., 1994). Recently, we have observed that PII also inhibits the kinase activity of NRII (P. Jiang and A. J. Ninfa, unpublished data). Thus, the binding of PII converts NRII from a kinase to a phosphatase. The phosphorylated form of NRI is an activator of transcription from nitrogen-regulated promoters that are transcribed by σ^{54}-RNA polymerase (Ninfa and Magasanik, 1986). PII prevents transcription from these promoters by bringing about the dephosphorylation of NRI∼P by NRII. In addition, PII activates the adenylylation activity of adenylyltransferase (ATase), product of *glnE*, which regulates the activity of glutamine synthetase (GS) by reversible adenylylation [Brown *et al*., 1971; Jiang *et al*., 1998a]. Glutamine synthetase is the most important enzyme for the assimilation of the preferred nitrogen source, ammonia, when ammonia is limiting. Adenylylation of a GS subunit results in its inactivation.

Much previous work, including studies in which the regulatory system was reconstituted from purified components, indicated that the key signal of nitrogen status is the concentration of glutamine, while the key signal of carbon status is the concentration of 2-ketoglutarate (Senior, 1975; Engleman and Francis, 1978; Jiang *et al*., 1998a,b). These effectors act antagonistically to regulate PII activity by different mechanisms (Kamberov *et al*., 1995; Liu and Magasanik, 1995; Jiang *et al*., 1998a,b). Glutamine regulates the activity of PII by controlling its uridylylation state and by controlling the interaction of PII with the ATase. PII is reversibly uridylylated by the signal-transducing uridylyltransferase/uridylyl-removing enzyme (UTase/UR, product of *glnD*); glutamine inhibits the uridylyltransferase reaction

and activates the uridylyl-removing reaction (Engleman and Francis, 1978; Jiang *et al*., 1998c). Thus, under conditions of nitrogen excess, when the internal glutamine concentration is high, PII is mainly unmodified, while in conditions of nitrogen limitation, PII is mainly uridylylated. In addition, glutamine activates the adenylylation reaction of ATase synergistically with PII. When the concentration of glutamine is high, a low concentration of PII is able to activate the adenylylation activity of ATase, whereas at low concentrations of glutamine, a higher concentration of PII is required to activate ATase (P. Jiang and A. J. Ninfa, unpublished). In the absence of PII, glutamine is a weak activator of the ATase adenylylation activity (Brown *et al*., 1971; Rhee *et al*., 1989).

The uridylylation of PII prevents its interaction with NRII, and thus prevents the dephosphorylation of NRI~P (Atkinson *et al*., 1994; Kamberov *et al*., 1995). PII~UMP is an essential activator for the deadenylylation activity of ATase (Brown *et al*., 1971; Rhee *et al*., 1989), which catalyses the conversion of the inactive GS~AMP to active GS.

The carbon signal, 2-ketoglutarate, regulates the activity of unmodified PII allosterically (Liu and Magasanik, 1995; Kamberov *et al*., 1995; Jiang *et al*., 1998a,b,c). The trimeric PII contains three sites that bind 2-ketoglutarate. However, binding of this effector to the three sites is not independent (Kamberov *et al*., 1995; Jiang *et al*., 1998c). The first molecule of 2-ketoglutarate binds to PII at a very low concentration (≈5 μM; Kamberov *et al*., 1995; Jiang *et al*., 1998a), such that PII is probably bound all the time *in vivo*, where the 2-ketoglutarate concentration is reported to range from 0.1 to 0.9 mM (Senior, 1975). Binding of additional molecules of 2-ketoglutarate to PII only occurs at higher concentrations, with dissociation constants estimated from kinetic studies to be approximately 50–250 μM (Jiang *et al*., 1998a,b,c). Thus, binding of the first molecule of 2-ketoglutarate to PII exerts negative co-operativity on the binding of additional effector molecules. PII trimers containing a single bound 2-ketoglutarate interact optimally with NRII and ATase (Kamberov *et al*., 1995; Liu and Magasanik, 1995; Jiang *et al*., 1998b,c). When the negative co-operativity is overcome at elevated concentrations of 2-ketoglutarate, the ability of PII to interact with NRII and ATase is diminished (Kamberov *et al*., 1995; Liu and Magasanik, 1995; Jiang *et al*., 1998a,b,c). Thus, growth in the presence of a good carbon source, resulting in a high intracellular concentration of 2-ketoglutarate, prevents the dephosphorylation (inactivation) of NRI~P and the adenylylation (inactivation) of GS.

Recent work has indicated that *E. coli* and several other bacteria contain multiple versions (paralogues) of the PII protein. In *E. coli*, GlnK, product of *glnK*, is a PII paralogue that plays a role in nitrogen regulation (Allikmets *et al*., 1993; van Heeswijk *et al*., 1995; 1996; Atkinson and Ninfa, 1998). Unlike PII, which is present at a constant level under all conditions, the expression of *glnK* is nitrogen regulated such that essentially no GlnK is found in nitrogen-replete cells, and a high intracellular concentration of GlnK is found in nitrogen-starved cells (van Heeswijk *et al*., 1996; Atkinson and Ninfa, 1998). Experiments with a *lacZ* transcriptional (operon) fusion to the *glnK* promoter indicate that this promoter is strongly expressed in nitrogen-starved cells (Atkinson and Ninfa, 1998). Under starvation conditions, GlnK may contribute to nitrogen regulation by controlling both ATase and NRII (Atkinson and Ninfa, 1998). Indeed, *E. coli* has a severe growth defect on minimal medium in the absence of exogenous amino acids if it lacks both GlnK and PII because, in such cells, the high expression of the Ntr regulon apparently results in the degradation of several amino acids (Atkinson and Ninfa, 1998). This high expression of the Ntr regulon is caused by the unregulated activity of NRII (Atkinson and Ninfa, 1998).

Genetic analysis of *glnK* indicated that, in cells containing PII, the loss of GlnK had little effect on the expression of the nitrogen-regulated *glnAp2* promoter and only a modest effect on the regulation of the *glnK* promoter (Atkinson and Ninfa, 1998). Also, the loss of GlnK had little effect on the regulation of the GS adenylylation state, as long as PII was present (Atkinson and Ninfa, 1998). Thus, PII plays the dominant role in the regulation of these phenotypes (Bueno *et al*., 1985). However, in cells deleted for PII, GlnK was responsible for regulation of these phenotypes, as already noted. In cells lacking PII, the regulation of the adenylylation state of GS by ammonia was wild type and required the presence of GlnK (Atkinson and Ninfa, 1998). In contrast, in cells lacking PII, the regulation of *glnA* expression by ammonia is eliminated, and the regulation of *glnK* expression by ammonia is defective. The residual regulation of *glnK* expression by ammonia is caused by GlnK (Atkinson and Ninfa, 1998). These observations suggested that GlnK could interact well with the ATase, but was not as effective as PII in activating the NRII phosphatase activity. Nevertheless, GlnK is required for the regulation of NRII in the absence of PII.

The regulation of GlnK activity by the UTase/UR is indicated by several observations. In the absence of the UTase/UR, but not in its presence, GlnK prevents the expression of some Ntr genes that apparently require a high level of NRI~P for expression (Atkinson and Ninfa, 1998). Also, the uridylylation of GlnK by the UTase/UR has been observed directly *in vivo* (van Heeswijk *et al*., 1996).

Cells lacking PII and the UTase/UR cannot fully activate the Ntr regulon, because of the presence of unmodified GlnK, which elicits enough of the NRII phosphatase activity to maintain the concentration of NRI~P below the threshold needed for activation of some Ntr genes. At least one

of the genes required for the use of arginine as a nitrogen source is not expressed in such cells, rendering the cells unable to grow when arginine is the sole nitrogen source. In contrast, the triply null *glnB glnD glnK* strain lacking UTase/UR, PII and GlnK grows poorly on arginine as the sole nitrogen source, because of its inability to prevent degradation of amino acids, but this growth is considerably better than that of its *glnK*$^+$ parent. Spontaneous mutations in *glnK* that permitted growth on arginine were isolated in the doubly mutant *glnB glnD* strain (Atkinson and Ninfa, 1998). Some of the *glnK* mutants obtained had growth properties suggesting that the *glnK* alleles were null, while other *glnK* mutants had properties that suggested that the altered GlnK was moderately defective in the interaction with NRII, and interacted normally with the UTase/UR and ATase.

We purified wild-type GlnK, the GlnKY51N mutant protein, which appeared to be null or nearly so in cells (*Experimental procedures*), and the GlnKR47W mutant protein, which appeared to be specifically defective in the interaction with NRII in cells (Atkinson and Ninfa, 1998), and compared their activities with those of PII using purified components. Surprisingly, GlnK was a potent activator of the phosphatase activity of NRII (about half as active as PII) but was a very poor activator of the adenylylation of glutamine synthetase by ATase (about 1/40th as active as PII). The GlnK protein was readily uridylylated by the UTase/UR. However, the deuridylylation of GlnK~UMP by the uridylyl-removing activity of the UTase/UR was very slow (about 1/10th the rate of PII~UMP deuridylylation). Thus, when reconstituted with the UTase/UR in a monocyclic system, the PII uridylylation state was effectively regulated by glutamine, as shown before, but GlnK uridylylation, while slowed by glutamine, did not reach a steady state. Thus, GlnK has unique properties that may illustrate its role in nitrogen regulation. We also observed that the GlnKR47W protein was specifically defective in the interaction with NRII *in vitro*, as predicted (Atkinson and Ninfa, 1998), while the GlnKY51N protein was essentially non-functional *in vitro*.

Results

Purification of GlnK, GlnKR47W and GlnKY51N

We engineered the overexpression of the desired *glnK* alleles and purified their products using the methods used previously for PII (Kamberov *et al*., 1994). A second preparation of GlnK was also used in our studies, prepared identically except for the last chromatography step (*Experimental procedures*). In each case, the purified proteins were about 90% or more pure, as estimated by SDS–PAGE (data not shown). As one of the purification steps involved gel filtration chromatography, we could readily observe that, like PII, GlnK and the mutant GlnK proteins appeared to be trimers (data not shown). The trimeric nature of GlnK was examined by examining the time course of GlnK uridylylation by the purified UTase/UR using non-denaturing gel electrophoresis, as described previously for PII (Atkinson *et al*., 1994). This analysis also suggested that GlnK was a trimer, as three distinct uridylylated species could be observed corresponding to GlnK molecules containing one, two or three uridylyl groups (data not shown). While our work was under way, the structure of the trimeric GlnK was solved (Xu *et al*., 1998)

Interaction of GlnK, GlnKR47W and GlnKY51N with NRII

To assess the interaction of GlnK with NRII, we measured the rate of NRI~P dephosphorylation in reaction mixtures containing NRI, NRII and ATP, as described previously (Fig. 1; Kamberov *et al*., 1994; 1995). During an initial phase of the reaction, NRI, present in excess, was phosphorylated by NRII and ATP. The rate of NRI~P dephosphorylation was then assessed after the addition of PII, GlnK or a buffer control. Previous results have shown that the rate of NRI~P dephosphorylation and the steady-state level of NRI phosphorylation in this assay is dependent on the PII concentration and on the 2-ketoglutarate concentration (Kamberov *et al*., 1994; 1995). In Fig. 1A,

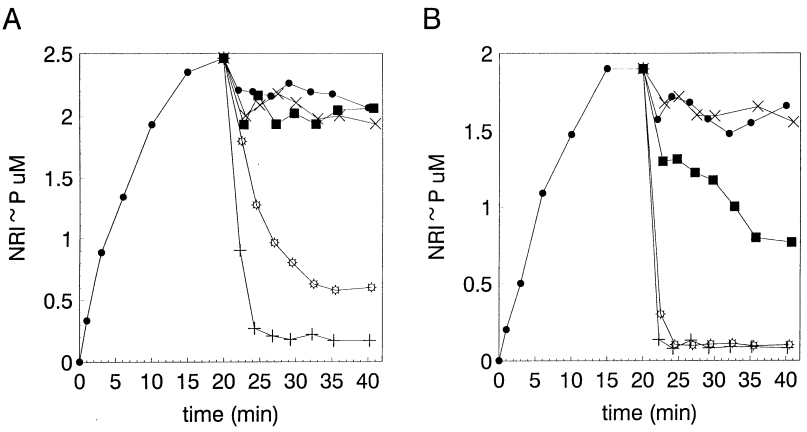

Fig. 1. Effect of PII, GlnK and GlnK* proteins on the dephosphorylation of NRI~P by NRII. Reactions were incubated at 25°C and contained 100 mM Tris-Cl, pH 7.5, 100 mM KCl, 25 mM $MgCl_2$, 300 μM 2-ketoglutarate, 300 μg ml^{-1} BSA, 600 μM [γ-^{32}P]-ATP, 18 μM NRI and 0.3 μM NRII. Reactions were incubated for 20 min to permit the phosphorylation of NRI by NRII, after which PII, GlnK or a GlnK* protein was added.
A. Effect of addition of PII, GlnK or GlnK* proteins to 0.3 μM. Symbols are: (+), PII; (*), GlnK; (filled square), GlnKR47W; (X), GlnKY51N; (filled circle), storage buffer control.
B. Effect of addition of PII, GlnK or GlnK* proteins to 3.0 μM. Symbols are as in (A).

typical results are shown for experiments with 2-ketoglutarate at the intermediate concentration of 300 μM and PII or GlnK at 0.3 μM, a limiting concentration for PII. As shown, PII brought about the rapid dephosphorylation of NRI~P, and GlnK clearly had activity, but was less effective. When present at this concentration, GlnKR47W and GlnKY51N did not have significant activity. In contrast, in Fig. 1B, typical results are shown for experiments at 300 μM 2-ketoglutarate and 3 μM PII or GlnK, which is a saturating concentration for PII. At this concentration, both PII and GlnK brought about the very rapid dephosphorylation of NRI~P, GlnKR47W was clearly less effective and GlnKY51N seemed to lack activity.

As elevated 2-ketoglutarate diminishes the interaction of PII with NRII (Liu and Magasanik, 1995; Kamberov *et al*., 1995; Jiang *et al*., 1998b), we examined the activity of GlnK at various 2-ketoglutarate concentrations. A low concentration of 2-ketoglutarate stimulated the ability of GlnK to activate the NRII phosphatase, whereas elevated 2-ketoglutarate inhibited the activity of GlnK (Fig. 2A, curves with symbols X, filled diamonds, filled triangles and house show the effects of 50 μM, 150 μM, 500 μM and 2 mM 2-ketoglutarate respectively). Under the conditions used, the inhibition of GlnK activation of the NRII phosphatase was more dramatic than that observed with PII (Fig. 2B). These results suggest that, like PII, GlnK is allosterically regulated by 2-ketoglutarate.

Activation of the adenylylation activity of ATase by PII, GlnK, GlnKR47W and GlnKY51N

Previous results have indicated that PII can activate the adenylylation of GS by ATase, even in the absence of 2-ketoglutarate (Brown *et al*., 1971; Jiang *et al*., 1997). For PII, this activity is greatly increased at low concentrations of 2-ketoglutarate and is prevented by 2-ketoglutarate at high concentrations (Jiang *et al*., 1998c). Also, the activation of ATase by PII (and 2-ketoglutarate) is synergistic with glutamine. At high glutamine concentrations, a low concentration of PII is able to activate the enzyme, while at low glutamine concentrations, a high concentration of PII is required to activate the ATase (P. Jiang and A. J. Ninfa, unpublished).

We examined first the activation of ATase by PII, GlnK, GlnKR47W and GlnKY51N in the absence of 2-ketoglutarate (Fig. 3). Under these conditions, PII was clearly the most potent activator, GlnKR47W and GlnK had clearly detectable activity and GlnKY51N appeared to lack activity (Fig. 3). Interestingly, GlnKR47W was a better activator of ATase then was wild-type GlnK (Fig. 3).

The ability of GlnK to activate the ATase in the presence of glutamine (6 mM) and 2-ketoglutarate (25 μM) was examined and compared with that of PII. Under these conditions, PII is a potent activator of ATase (Jiang *et al*., 1998c). When the initial rate of GS adenylylation was examined, 20 μM GlnK was required to attain an initial rate of GS adenylylation similar to that obtained with 0.5 μM PII (data not shown). Thus, while GlnK could activate ATase under these conditions, it was about 40-fold less potent than PII.

The ability of purified GlnK to activate the adenylylation of GS by ATase has previously been studied indirectly by measurement of the activity of GS after treatment with the ATase ± GlnK or PII (van Heeswijk *et al*., 1996). In that study, GlnK was also observed to be approximately 1/40th as active as PII in activating the adenylylation of GS by ATase.

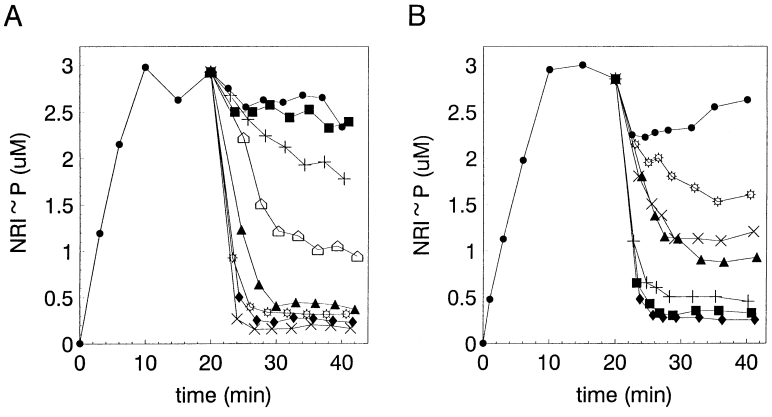

Fig. 2. Effect of 2-ketoglutarate concentration on the activation of the NRII-catalysed dephosphorylation of NRI~P by PII and GlnK. Reaction conditions were as in Fig. 2, except that 2-ketoglutarate was varied as indicated below.
A. The symbols are: (X), 1 μM GlnK and 50 μM 2-ketoglutarate; (filled diamond), 1 μM GlnK and 150 μM 2-ketoglutarate; (filled triangle), 1 μM GlnK and 500 μM 2-ketoglutarate; (house), 1 μM GlnK and 2 mM 2-ketoglutarate; (filled square) 1 μM GlnK and 0 2-ketoglutarate; (asterisk), 300 nM PII and 50 μM 2-ketoglutarate; (+) 300 nM PII and 0 2-ketoglutarate; (filled circle), storage buffer control.
B. The symbols are: (filled diamond), 300 nM PII and 550 μM 2-ketoglutarate; (filled square), 300 nM PII and 850 μM 2-ketoglutarate; (+) 300 nM PII and 2 mM 2-ketoglutarate; (filled triangle), 300 nM GlnK and 500 μM 2-ketoglutarate; (X) 300 nM GlnK and 850 μM 2-ketoglutarate; (asterisk), 300 nM GlnK and 2 mM 2-ketoglutarate; (filled circle), storage buffer control.

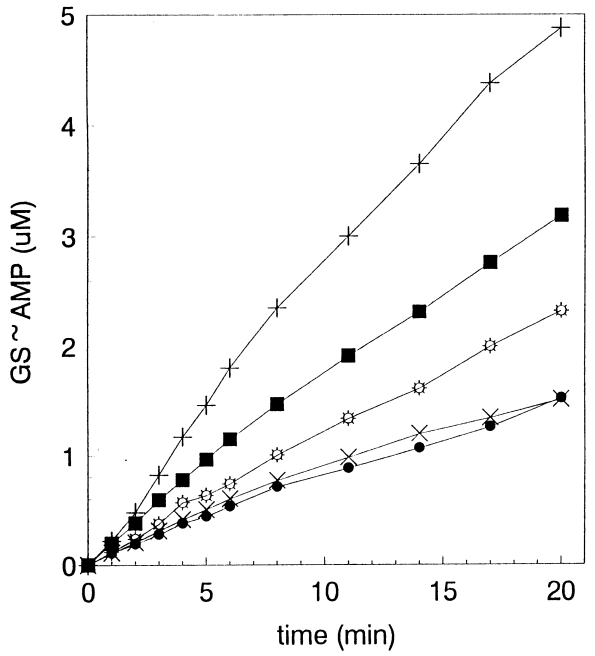

Fig. 3. Activation of GS adenylylation by PII, GlnK and the GlnK* proteins. Reactions were incubated at 30°C and contained 100 mM Tris-Cl, pH 7.5, 25 mM $MgCl_2$, 300 μg ml^{-1} BSA, 5 mM glutamine, 500 μM [α-^{32}P]-ATP, 10 nM ATase and 1 μM GS (dodecamers). The symbols are: (+) 400 nM PII; (filled square) 400 nM GlnKR47W; (asterisk) 400 nM GlnK; (X) 400 nM GlnKY51N; (filled circle), storage buffer control.

The ability of PII to activate the adenylylation activity of ATase is stimulated by very low concentrations of 2-ketoglutarate and is inhibited by higher concentrations of 2-ketoglutarate (Jiang *et al*., 1998c). Previous work has established that all 2-ketoglutarate effects on the PII-activated ATase adenylylation reaction are caused by the binding of 2-ketoglutarate to PII (Jiang *et al*., 1998c). We therefore examined whether GlnK was similarly allosterically regulated by 2-ketoglutarate.

The adenylylation of GS by ATase was examined in reaction mixtures containing saturating glutamine (6 mM), fixed GlnK (25 μM) and various concentrations of 2-ketoglutarate (Fig. 4). The highest rate of GS adenylylation was obtained in reaction mixtures lacking 2-ketoglutarate and, at elevated concentrations of 2-ketoglutarate, the adenylylation of GS was inhibited considerably (Fig. 4). Thus, GlnK seems to be regulated allosterically by 2-ketoglutarate, like PII, but the activation of adenylylation at low concentrations of effector that was observed previously with PII was not observed with GlnK.

Experiments with intact cells lacking PII have shown that GlnK can mediate the regulation of the GS adenylylation state (Atkinson and Ninfa, 1998). In starved cells lacking PII but containing GlnK, the GS adenylylation state is low when nitrogen is limiting, and is rapidly increased if the cells are shifted to medium containing ammonia (van Heeswijk *et al*., 1996; Atkinson and Ninfa, 1998). We examined whether the regulation of ATase by glutamine, in synergy with GlnK, could explain this phenomenon. Reaction mixtures initially contained ATase, GS and either GlnK, a mixture of GlnK and GlnK~UMP, or a buffer control. In all cases, negligible adenylylation of GS was observed (Fig. 5). Glutamine (6 mM) was then added to each reaction mixture; this addition resulted in increased adenylylation of GS in all cases, and the rate of GS adenylylation was greatly increased by GlnK and by the combination of GlnK and GlnK~UMP (Fig. 5). This experiment suggests that synergy between GlnK and glutamine in the activation of ATase, or the activation of ATase by glutamine alone, may explain the earlier results obtained with intact cells.

Uridylylation and deuridylylation of PII, GlnK, GlnKR47W and GlnKY51N

Previous results have indicated that the uridylylation of PII by the UTase/UR requires 2-ketoglutarate at low concentrations, corresponding to the concentration resulting in the binding of a single molecule of this effector to PII, and was not greatly inhibited by elevated concentrations of this effector (Kamberov *et al*., 1995; Jiang *et al*., 1998a). The rate of uridylylation was examined in experiments in which PII or GlnK was present in large excess (10 μM), enzyme was limiting (15 nM) and 2-ketoglutarate was present at 300 μM. Under these conditions, the rate of PII uridylylation was linear for 4 min (Fig. 6, + symbols), and glutamine at the intermediate concentration of 125 μM brought about a significant decrease in the rate of PII uridylylation (Fig. 6, × symbols). GlnK was uridylylated nearly as well as PII (Fig. 6, squares), and the rate of GlnK uridylylation was also diminished in the presence of glutamine (Fig. 6, circles).

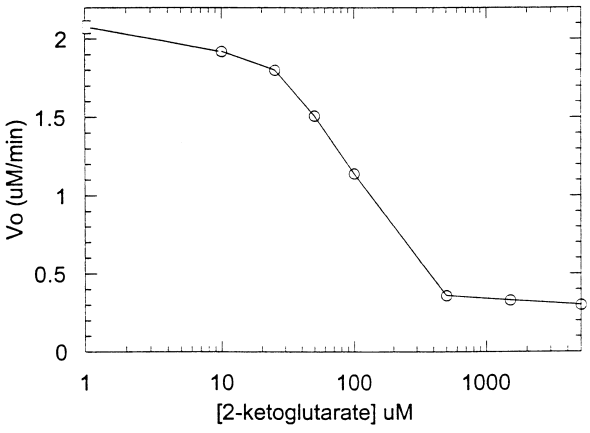

Fig. 4. Effect of 2-ketoglutarate concentration on the stimulation of GS adenylylation by GlnK. Initial rates were determined in reactions that contained 100 mM Tris-Cl, pH 7.5, 100 mM KCl, 1 mM DTT, 10 mM KPi, 25 mM $MgCl_2$, 300 μg ml^{-1} BSA, 6 mM glutamine, 500 μM [α-^{32}P]-ATP, 50 nM ATase, 1 μM GS (dodecamers), 25 μM GlnK and various concentrations of 2-ketoglutarate.

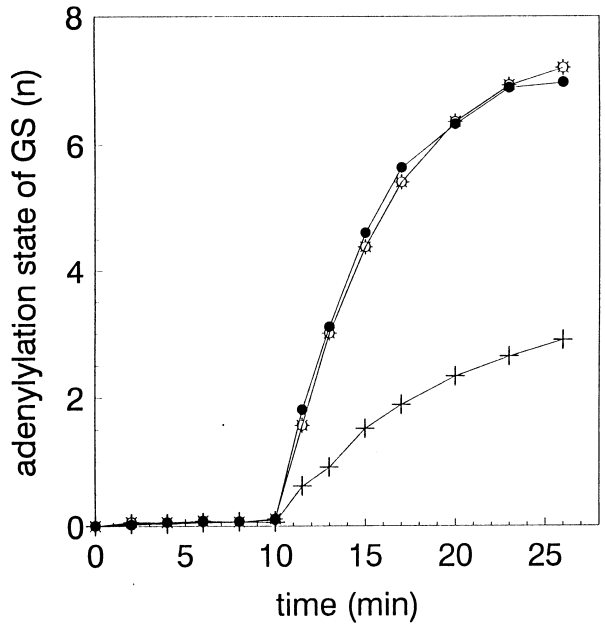

Fig. 5. Synergistic stimulation of the adenylylation activity of ATase by GlnK and glutamine. Reactions were performed at 30°C and contained 100 mM Tris-Cl, pH 7.5, 100 mM KCl, 25 mM $MgCl_2$, 1 mM DTT, 300 μg ml^{-1} BSA, 100 μM 2-ketoglutarate, 10 mM KPi, 1 μM GS (dodecamers) and 500 μM [α-^{32}P]-ATP. GlnK or both GlnK and GlnK~UMP were present from the beginning of the experiment, as indicated. After 10 min of incubation, glutamine was added to the reaction mixtures to 6 mM. Symbols are: (+) no GlnK or GlnK~UMP added; (asterisk), 25 μM GlnK and 10 μM GlnK~UMP added; (filled circle), 25 μM GlnK added.

Interestingly, in the presence of glutamine, GlnK was uridylylated slightly faster than PII. The uridylylation of GlnKR47W (Fig. 6, triangles) was slower than that of GlnK and was also decreased by glutamine (Fig. 6, wedges). We could not detect the uridylylation of GlnKY51N (data not shown), as expected, as position 51 is the site of PII uridylylation (Jiang *et al*., 1997). In additional experiments, we observed that the uridylylation of GlnK and GlnKR47W required 2-ketoglutarate, and PII uridylylation was barely detectable in the absence of 2-ketoglutarate (data not shown).

The K_{act} for 2-ketoglutarate activation of uridylylation was examined for PII and GlnK. Previous results had indicated that, for PII, the 2-ketoglutarate K_{act} corresponded well to the K_d for the dissociation of 2-ketoglutarate from PII bound to a single molecule of this effector (Jiang *et al*., 1998a). The K_{act} for PII uridylylation was about 4 μM, in good agreement with earlier results (Jiang *et al*., 1998a), whereas that for GlnK uridylylation was about 7–15 μM 2-ketoglutarate (Fig. 7).

To examine the deuridylylation of GlnK~UMP by the UR activity of the UTase/UR, we purified PII~UMP and GlnK~UMP as described previously (Jiang *et al*., 1998a). Under conditions of saturating glutamine (6 mM) and 100 μM 2-ketoglutarate, the initial rate of deuridylylation of GlnK~UMP (Fig. 8, wedge) was about 10-fold slower than the rate of PII~UMP deuridylylation (Fig. 8, +). At very high enzyme concentrations, GlnK~UMP could be slowly deuridylylated (Fig. 8, filled wedge). In additional experiments, we examined the effect of variation in the 2-ketoglutarate concentration on the rate of GlnK~UMP deuridylylation. We observed that, as shown earlier for PII~UMP deuridylylation (Jiang *et al*., 1998a), 100 μM 2-ketoglutarate was nearly saturating, and the rate of GlnK~UMP deuridylylation was not significantly increased at higher 2-ketoglutarate concentrations (data not shown). These experiments demonstrate that the deuridylylation of GlnK~UMP was very slow under conditions in which PII~UMP was readily deuridylylated.

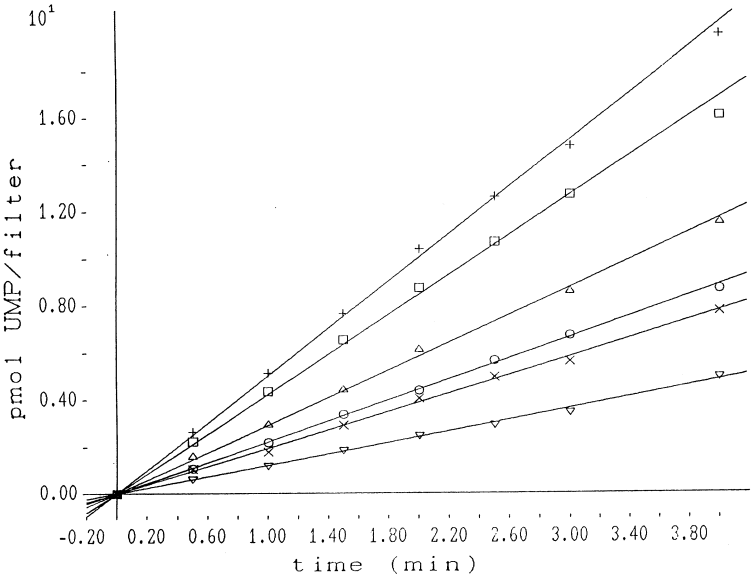

Fig. 6. Uridylylation of PII, GlnK and GlnKR47W by the UTase activity of the UTase/UR, and inhibition of uridylylation by glutamine. Reactions were incubated at 30°C and contained 100 mM Tris-Cl, pH 7.5, 100 mM KCl, 25 mM $MgCl_2$, 1 mM DTT, 300 μM 2-ketoglutarate, 500 μM ATP, 500 μM [α-^{32}P]-UTP and 15 nM UTase/UR. Symbols are: (+), 10 μM PII; (square), 10 μM GlnK; (triangle), 10 μM GlnKR47W; (circle), 10 μM GlnK + 125 μM glutamine; (X), 10 μM PII + 125 μM glutamine; (wedge), 10 μM GlnKR47W + 125 μM glutamine.

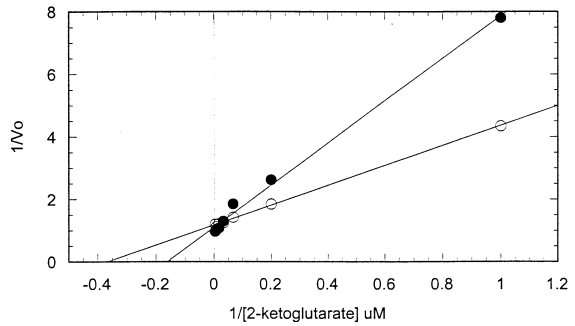

Fig. 7. K_{act} for 2-ketoglutarate activation of uridylylation of PII and GlnK. Initial rates were determined in reactions performed at 30°C containing 100 mM Tris-Cl, pH 7.5, 100 mM KCl, 500 μM ATP, 25 mM $MgCl_2$, 1 mM DTT, 300 μg ml^{-1} BSA, 400 μM [α-^{32}P]-UTP, 10 nM UTase/UR, either 15 μM PII or 15 μM GlnK and the indicated 2-ketoglutarate concentrations. Symbols are: (filled circle), GlnK; (open circle), PII.

In additional experiments, we examined whether GlnK~UMP inhibited the deuridylylation of PII~UMP by the UR activity of the UTase/UR. For these experiments, PII~UMP was present at 2 μM, which corresponds approximately to the K_m for this reaction (Jiang *et al*., 1998a). Under these conditions, GlnK~UMP was a poor inhibitor of PII~UMP deuridylylation (data not shown). We could not add a high enough concentration of GlnK~UMP to reaction mixtures to obtain 50% inhibition of the rate of PII~UMP deuridylylation (data not shown), therefore K_i must be very high.

Reconstitution of the UTase/UR-GlnK monocycle

The regulation of the steady-state level of PII and GlnK uridylylation by glutamine was examined in reaction mixtures that initially contained unmodified PII or GlnK at 1.25 μM, UTase/UR at 0.1 μM and 2-ketoglutarate at 200 μM. Previous studies have shown that, under these conditions, PII uridylylation rapidly reaches a steady-state level that is dependent on the glutamine concentration (Jiang *et al*., 1998b); as a control, these experiments were repeated (Fig. 9A). In contrast, GlnK uridylylation state never reached a steady state under similar conditions, unless GlnK was completely modified (Fig. 9B). While glutamine slowed the rate of GlnK uridylylation, eventually GlnK became highly uridylylated even in the presence of elevated glutamine concentrations.

Discussion

Biochemical characterization of purified GlnK cannot prove the function of GlnK in intact cells, but may serve to limit the possibilities. The biochemical studies presented here and the genetic studies presented previously (Atkinson and Ninfa, 1998) indicate that PII and GlnK have distinct properties, which probably reflect their unique roles in regulating nitrogen assimilation. PII, which is present at a constant, relatively low concentration, was a potent activator of NRII and ATase, and its interaction with the UTase/UR permitted efficient regulation of its uridylylation state by glutamine. As the interaction of PII with the NRII and ATase receptors is regulated allosterically by 2-ketoglutarate, PII is ideally suited to integrate information on the carbon and nitrogen status of the cell and to regulate these receptors accordingly (Jiang *et al*., 1998b,c).

GlnK, on the other hand, is not present at a constant concentration in the cell; the activation of *glnK* transcription by NRI~P places the appearance of GlnK under the control of PII and, in starved cells, under the control of

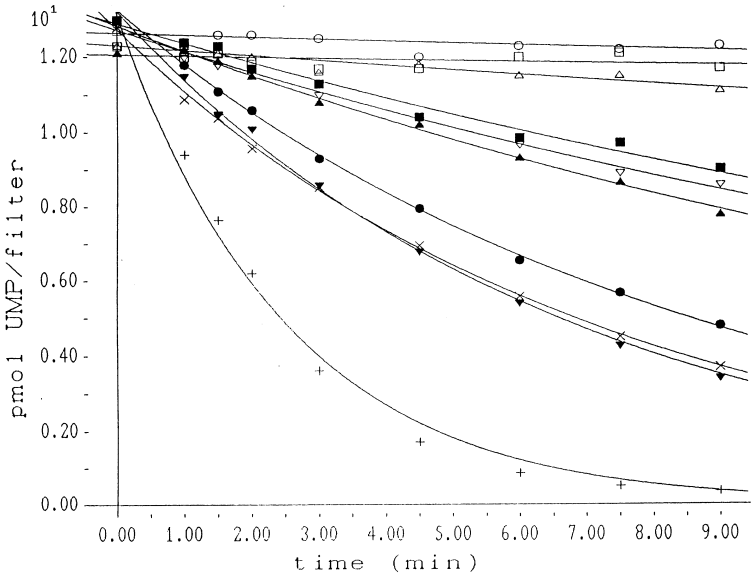

Fig. 8. Deuridylylation of PII~UMP and GlnK~UMP by the UR activity of the UTase/UR. Reactions were performed at 30°C and contained 100 mM Tris-Cl, pH 7.5, 100 mM KCl, 25 mM $MgCl_2$, 1 mM DTT, 100 μM 2-ketoglutarate, 500 μM ATP, 300 μg ml^{-1} BSA and either 3.2 μM [^{32}P]-GlnK~UMP or [^{32}P]-PII~UMP. The symbols are: (circle), GlnK~UMP, 6 mM glutamine, no enzyme; (square), PII~UMP, 6 mM glutamine, no enzyme; (triangle), GlnK~UMP, 1 μM UTase/UR; (filled square), GlnK~UMP, 3 μM UTase/UR; (wedge), GlnK~UMP, 6 mM glutamine, 1 μM UTase/UR; (filled triangle), GlnK~UMP, 5 μM UTase/UR; (filled circle), GlnK~UMP, 6 mM glutamine, 3 μM UTase/UR; (X), PII~UMP, 1 μM Utase/UR; (filled wedge) GlnK~UMP, 6 mM glutamine, 5 μM UTase/UR; (+), PII~UMP, 6 mM glutamine, 1 μM UTase/UR.

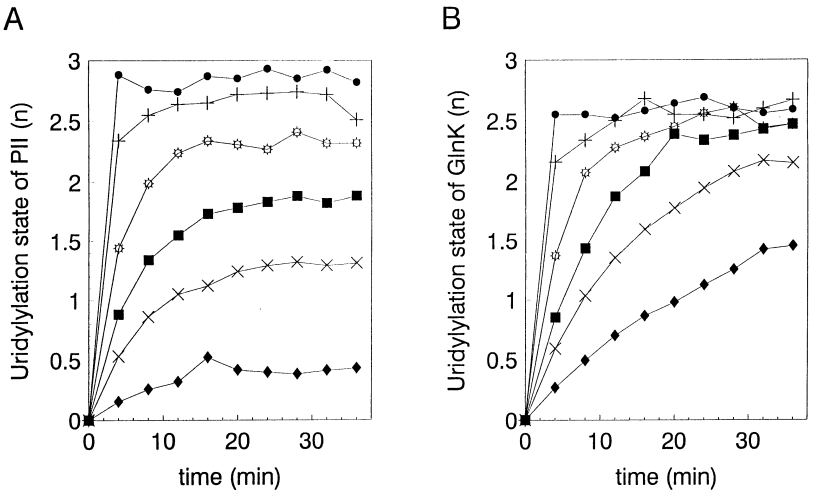

Fig. 9. Regulation of uridylylation state by glutamine in reconstituted PII-UTase/UR and GlnK-UTase/UR monocycles. All reactions were performed at 30°C in 100 mM Tris-Cl, pH 7.5, 100 mM KCl, 25 mM $MgCl_2$, 500 μM ATP, 200 μM 2-ketoglutarate, 1 mM DTT, 500 μM [α-^{32}P]-UTP, 300 μg ml^{-1} BSA and 100 nM UTase/UR.
A. 1.25 μM PII was used.
B. 1.25 μM GlnK was used.
Symbols are: (filled diamonds) 4 mM glutamine; (X) 1.2 mM glutamine; (filled squares), 0.8 mM glutamine; (asterisk), 0.5 mM glutamine; (+), 0.25 mM glutamine; (filled circle) 0 glutamine.

GlnK (van Heeswijk *et al*., 1996; Atkinson and Ninfa, 1998). As PII is a potent activator of the NRII phosphatase activity, GlnK probably never accumulates in the cell until most of the PII has been converted to PII∼UMP. Previous results with a *glnK::lacZ* transcriptional fusion suggested that, upon nitrogen starvation, the *glnK* gene is strongly transcribed (Atkinson and Ninfa, 1998). As the intracellular concentration of glutamine is low under these conditions, there will be little inhibition of the transferase activity of the UTase/UR, and GlnK will become uridylylated. However, the presence of both GlnK and GlnK∼UMP in nitrogen-starved cells has been observed in two separate studies (van Heeswijk *et al*., 1996; He *et al*., 1998). Thus, it is possible that, when the rate of GlnK synthesis becomes sufficiently high, the fixed level of UTase activity cannot keep up, and both GlnK and GlnK∼UMP will be present. Indeed, the ratio of GlnK to GlnK∼UMP may increase to a fixed maximum as cells become starved.

As GlnK is only present in nitrogen-starved cells, it must provide some function beneficial for such cells. The presence of unmodified GlnK in starved cells would ensure that there is always some NRII phosphatase activity. We observed that GlnK was a potent activator of the NRII phosphatase activity (Fig. 1). One hypothesis for the role of GlnK is that it contributes to the regulation of NRII under nitrogen-limiting conditions. Consistent with this hypothesis, previous genetic studies have indicated that, in the absence of both PII and GlnK, the presence of NRII resulted in unrestrained Ntr gene expression, which can be catastrophic under certain conditions (Atkinson and Ninfa, 1998). Also, in the absence of PII, GlnK clearly acts through NRII to regulate its own promoter and other Ntr promoters (Atkinson and Ninfa, 1998). However, there is a problem with this hypothesis. Previous genetic studies failed to identify growth conditions in which GlnK was of benefit to cells containing PII (Atkinson and Ninfa, 1998). If PII is entirely converted to PII∼UMP in starved cells, GlnK should be required to maintain control of NRII. Yet, cells containing PII and lacking GlnK survive starvation and the shift to nitrogen-limiting conditions as well as do isogenic cells containing GlnK (Atkinson and Ninfa, 1998). Thus, while not necessary for the regulation of NRII in cells containing PII, GlnK may have a subtle role in fine-tuning NRII activity in starved cells.

GlnK was about 40-fold less potent than PII in activating the adenylylation of GS by ATase. This result is consistent with the earlier observation that purified GlnK was ineffective in bringing about the inactivation of GS by ATase (van Heeswijk *et al*., 1996). While other explanations cannot be ruled out, these observations suggest that GlnK may not play a significant role in regulating ATase in starved cells containing PII. As PII∼UMP is present in such cells, a possible role for GlnK would be to facilitate the adenylylation of GS if ammonia were encountered. We observed that, like PII, GlnK can act synergistically with glutamine to activate the adenylylation of GS by ATase (Fig. 5). However, previous experiments have indicated that the rate of GS adenylylation in response to ammonia shock of starved cells (containing PII) is the same in the presence and in the absence of GlnK (Atkinson and Ninfa, 1998). Apparently, in cells lacking GlnK, the rapid deuridylylation of PII∼UMP in response to ammonia shock of starved cells is adequate to ensure the rapid adenylylation of GS (Atkinson and Ninfa, 1998). Alternatively, the activation of ATase by glutamine may be sufficient to account for the rapid adenylylation of GS under these conditions (Fig. 5).

Experiments with intact cells indicated that, in the absence of PII, GlnK was required for the regulation of the adenylylation state of GS (Atkinson and Ninfa, 1998). In such cells, the GlnK requirement is probably the result of a requirement for GlnK∼UMP for activation of the deadenylylation of GS∼AMP by ATase. The observation that cells

lacking PII could adenylylate GS rapidly in response to ammonia shock led us to the conclusion that the GlnK uridylylation state was rapidly regulated by the UTase/UR (Atkinson and Ninfa, 1998). However, the current study with purified components indicated that the uridylylation state of GlnK was not rapidly regulated by UTase/UR; rather, the deuridylylation of GlnK~UMP was very slow (Fig. 8). Thus, the rapid deuridylylation of GlnK~UMP cannot account for the ability of starved cells lacking PII to adenylylate GS rapidly in response to ammonia shock.

In our experiments, GlnK~UMP was deuridylylated slowly by the UR activity of the UTase/UR, and GlnK~UMP was a poor inhibitor of PII~UMP deuridylylation. This may be advantageous to the cell. If a starved wild-type cell (containing GlnK, GlnK~UMP and PII~ UMP) suddenly encounters ammonia, the internal glutamine concentration will rise rapidly. The UTase activity will become inhibited, and the UR activity will become activated. It may then be advantageous for the cell to deuridylylate PII~UMP preferentially, providing the highly potent PII protein to activate the NRII phosphatase activity and ATase adenylylation activity. The difference in potency between PII and GlnK and the fact that PII is synthesized constitutively ensures that, once PII~UMP is deuridylylated, the resulting PII dominates nitrogen regulation.

Allosteric regulation of GlnK activity by 2-ketoglutarate

Earlier studies with reconstituted systems have indicated that PII is regulated allosterically by 2-ketoglutarate, with different consequences for each receptor interaction. A low concentration of this effector stimulates the interaction of PII with each of its receptors. However, at higher effector concentrations, the interaction with ATase is strongly inhibited, the interaction with NRII is inhibited to a lesser extent and the interaction with the UTase/UR is not significantly inhibited (Jiang *et al*., 1998a,b,c). Apparently, PII adopts distinct conformations, depending on its degree of saturation with 2-ketoglutarate.

Our previous experiments with PII suggested that the K_{act} for the activation of PII uridylylation by 2-ketoglutarate corresponded to the K_d for the binding of a single effector molecule to the PII trimer (≈5 μM; Kamberov *et al*., 1995; Jiang *et al*., 1998a; this study). Here, we observed that the activation of GlnK uridylylation occurred at approximately 7–15 μM, suggesting that GlnK also binds an effector molecule with high affinity.

The activation of NRII and ATase by GlnK was inhibited by high concentrations of 2-ketoglutarate, similar to earlier observations with PII (Liu and Magasanik, 1995; Kamberov *et al*., 1995; Jiang *et al*., 1998a). Therefore, it seems that, like PII, the binding of additional effector molecules at elevated effector concentrations results in another conformation that does not interact well with NRII and ATase. However, there was a clear difference in the activation of the ATase by PII and GlnK. In this reaction, low concentrations of 2-ketoglutarate resulted in significant stimulation of GS adenylylation by PII (Jiang *et al*., 1998c), but not by GlnK. Thus, the binding of the first molecule of 2-ketoglutarate had different effects on the conformation of PII and GlnK, as reported by the ATase.

An altered form of GlnK specifically defective in the interaction with NRII

Experiments with PII have indicated that the apex of the T-loop (Carr *et al*., 1996) is essential for the interaction of PII with its three protein receptors (UTase/UR, NRII and ATase), and that mutations in this part of PII may have different effects on each of these interactions (Jiang *et al*., 1997). For instance, a PII mutation, A49P, resulted in an altered protein specifically defective in the interaction with NRII (Jiang *et al*., 1997). Our earlier genetic experiments suggested that *glnK* mutations resulting in the conversion of R-47 to a number of other amino acids may also result in proteins specifically defective in the interaction with NRII (Atkinson and Ninfa, 1998). Here, one such GlnK protein containing the alteration R47W was observed to be specifically defective in the interaction with NRII in experiments with purified components. This protein was uridylylated by the UTase/UR nearly as well as wild-type GlnK and activated the ATase more effectively than wild-type GlnK, but was defective in the activation of NRII. Thus, as proposed from genetic experiments (Atkinson and Ninfa, 1998), R-47 of GlnK seems to be very important for the interaction of GlnK with NRII.

In our experiments, the GlnK-Y51N protein appeared essentially to lack the ability to activate NRII and ATase. This was somewhat surprising, as earlier studies with this protein in intact cells indicated that it was able to bring about the relief of NifL-mediated inhibition of NifA (He *et al*., 1998; see below).

In addition to its role in the regulation of NRII and ATase, GlnK may interact with additional receptors

The synthesis of nitrogenase in *Klebsiella pneumoniae* requires the transcriptional activator NifA and is regulated by the NifL signal transduction protein. Nitrogenase synthesis is repressed at conditions of nitrogen sufficiency, reflecting the fact that the *nifLA* operon is an Ntr operon requiring a high intracellular concentration of NRI~P for transcription (Wong *et al*., 1987). However, recent work in which the *nifL* and *nifA* genes from *K. pneumoniae* were introduced into *E. coli* along with a reporter gene fusion to a NifA-activated gene (*nifH::lacZ*) indicated that nitrogen starvation and transcription of the Ntr regulon was required for relief of NifA from NifL-mediated inactivation,

even when the synthesis of NifL and NifA did not depend on NRI~P (He *et al*., 1997). This requirement is caused by the need for the GlnK protein, which somehow prevents the inactivation of NifA by NifL (He *et al*., 1998). Interestingly, GlnK-Y51N was also able to bring about the activation of NifA (He *et al*., 1998). While *E. coli* does not typically contain NifL and NifA, the regulation of these by GlnK raises the possibility that *E. coli* contains additional unknown receptors that are regulated by GlnK.

Experimental procedures

Bacteriological techniques

The bacterial strains and plasmids used in this study are summarized in Table 1. Rich LB + 0.2% (w/v) glutamine medium and W-salts minimal medium were prepared as described previously (Bueno *et al*., 1985). Ampicillin, when present, was at 100 μg ml^{-1}. The preparation of competent cells and transformation of competent cells with plasmid DNA was performed as described previously (Maniatis *et al*., 1982) Strains containing pJLA503-based plasmids were transformed and maintained at 30°C, as before (Kamberov *et al*., 1994).

Plasmid constructions

The overexpression of GlnK has been reported previously (van Heeswijk *et al*., 1996). However, analysis of the reported cloning method suggested that several amino acids from the *lac*α gene of the cloning vector had been added to the N-terminus of GlnK. When we reconstructed the GlnK overexpression plasmid as described previously, forming plasmid pKOP3, we observed that induction resulted in the expression of a protein that was significantly more massive than PII (data not shown). Yet, uninduced cultures containing this plasmid were reported to produce native-length GlnK (van Heeswijk *et al*., 1996). To simplify matters, pJLA503-based overexpression plasmids pKOP2 (wild-type GlnK), pKOPR47W (*glnK4*) and pKOPY51N (*glnK5*) were constructed as follows. The desired *glnK* alleles were obtained by polymerase chain reaction (PCR) amplification of chromosomal DNA from strains YMC10, OB25 and OB133 (Atkinson and Ninfa, 1998), according to the method of Saiki *et al*. (1990). The PCR primers used in all three cases were: (upstream primer) 5′-CGAATTCCATATG-AAGCTGGTGACCGTGAQTAATC and (downstream primer) 5′-CGGATCCGTCGACTTCCTGTTGCTGTGTGCCAGAG. These primers introduce a unique *Nde*I site overlapping the first ATG codon of *glnK* and a unique *Bam*HI site immediately downstream from the *glnK* coding sequence. The PCR products were purified using a Qiagen PCR purification kit, digested with *Nde*I and *Bam*HI, and cloned into similarly cleaved pJLA503. The plasmids were sequenced using the Oncor Fidelity DNA sequencing system to ensure that additional mutations were not introduced during PCR amplification. Plasmid DNA was prepared for DNA sequencing using the Qiagen plasmid miniprep kit.

Plasmid pKOP3 is a reconstruction of pWVH149 (van Heeswijk *et al*., 1996). It was constructed by cloning the *Eco*RI/*Eae*I fragment containing *glnK*$^+$ from pDK4 (Atkinson and Ninfa, 1998) into *Eco*RI- and *Not*I-digested pBluescript-II KS+ (Stratagene).

*Characterization of the phenotype of strain OB113 (*ΔglnB glnD99*::Tn*10 glnK5/*pgln2; Atkinson and Ninfa, 1998)*

To determine whether the *glnK5* allele was null, we compared the growth of strain OB113 (Atkinson and Ninfa, 1998) with the wild-type and control strains that were genetically identical (*ΔglnB glnD99::Tn10*/pgln2), except containing either *glnK*$^+$ or *Δmdl-glnK* (Atkinson and Ninfa, 1998). Cells were plated for single colonies on various solid media, and growth was observed after 72 h at 37°C. Media used were: glucose–ammonia–glutamine–tryptophan–Xgal (GNglnXtrp),

Table 1. Bacterial strains and plasmids used in this study.

Strain	Relevant genotype	Source/reference
JM109	Wild type	Gibco BRL
YMC10	Wild type	Backman *et al*. (1981)
BD	*ΔglnB glnD99::Tn10*	Atkinson and Ninfa (1998)
OB25	*ΔglnB glnD99::Tn10glnK4*/pgln62	Atkinson and Ninfa (1998)
OB113	*ΔglnB glnD99::Tn10glnK5*/pgln2	Atkinson and Ninfa (1998)
BDK	*ΔglnB glnD99::Tn10Δmdl-glnK::kan*	Atkinson and Ninfa (1998)
Plasmid	**Property/construction**	**Source/reference**
pJLA503	Hyperexpression vector	Schauder *et al*. (1987)
pKOP2	PCR *glnK*$^+$ *Nde*I/*Bam*HI into pJLA503	This study
pKOPR47W	PCR *glnK4 Nde*I/*Bam*HI into pJLA503	This study
pKOPY51N	PCR *glnK5 Nde*I/*Bam*HI into pJLA503	This study
pKOP3	Reconstruction of pWVH149 (van Heeswijk *et al*., 1996) *Eco*RI/*Eae*I of pDK4 into *Eco*RI/*Not*I cleaved pBluescript-II KS+	This study
pDK4	*Bam*HI of Kohara 149 into pUC18	Atkinson and Ninfa (1998)
pBluescript-II KS+	Hyperexpression vector	Stratagene
pgln2	*glnALG* operon (*Hin*dIII fragment) in pBR322	Backman *et al*. (1981)

glucose–glutamine–tryptophan–Xgal (GglnXtrp), glucose–ammonia (GN) and glucose–arginine (Garg), as described previously (Atkinson and Ninfa, 1998). All media also contained 0.004% (w/v) vitamin B1.

On GNglnXtrp, GglnXtrp and GN media, the wild-type strain YMC10 grew well, as did strains BD (*ΔglnB glnD99::Tn10*) and BD/pgln2 (*ΔglnB glnD99::Tn10*/pgln2). In contrast, strains BDK (*ΔglnB glnD99::Tn10Δmdl-glnK*) and BDK/pgln2 (*ΔglnB glnD99::Tn10Δmdl-glnK*/pgln2) only formed pinpoint colonies. Strain OB113 formed colonies that were intermediate in size between those formed by BD/pgln2 and BDK/pgln2. On Garg medium, OB113 grew as well as the wild type, whereas its parent BD/pgln2 (*ΔglnB glnD99::Tn10*/pgln2) did not grow at all. These results with intact cells indicate that, while the *glnK5* mutation (Y51N) results in a greatly altered GlnK protein, this mutation is not null.

GlnK hyperexpression and purification

To check protein induction using pKOP3 and to determine whether a fusion protein was programmed by this vector, cultures were grown overnight in LB + glutamine + ampicillin ± 1 mM IPTG at 37°C with vigorous aeration. Cells from a 1 ml aliquot of each culture were harvested by centrifugation, resuspended in 100 μl of 4× cracking buffer/dye solution, heated to 95°C for 15 min and subjected to electrophoresis on a 15% SDS–polyacrylamide gel as described previously (Kamberov *et al*., 1994). After electrophoresis, the gel was stained with Coomassie brilliant blue R250.

To check induction of pKOP2 in strain BD (Atkinson and Ninfa, 1998), a 2 ml LB + glutamine + ampicillin culture was grown overnight at 30°C with vigorous aeration. In the morning, a 1 ml aliquot of the overnight culture was used to inoculate 10 ml of fresh media, and this culture was grown for 1 h, after which it was split into two portions, which were grown at 30°C (non-inducing) and 43°C (inducing) for 3 h with vigorous aeration. Cells from 1 ml aliquots of the uninduced and induced cultures were harvested by centrifugation and analysed by SDS–PAGE as described above.

To grow large-scale batch cultures of cells for protein purification, strain BD containing either pKOP2, pKOPR47W or pKOPY51N was grown overnight in 2 ml of LB + glutamine + ampicillin medium at 30°C with vigorous aeration. This overnight culture (1 ml) was expanded to 500 ml using the same medium and growth conditions. After overnight growth, the 500 ml culture was divided equally into eight 2 l flasks, each containing 500 ml of fresh medium, and the culture was incubated at 30°C for 1 h, after which the incubation was at 44°C for 4 h with vigorous aeration. Cells were harvested by centrifugation, and cell pellets were stored at −80°C.

GlnK and GlnKR47W were purified using the method described previously for PII (Kamberov *et al*., 1994), with modifications as noted below. The overall scheme included three chromatography steps: DE52, Sephadex-G75 and hydroxylapatite, as before (Kamberov *et al*., 1994). Ammonium sulphate fractionation, used previously for purification of PII (Kamberov *et al*., 1994), was not used. Instead, ammonium sulphate precipitation at 50% saturation was used only to concentrate GlnK before loading the G75 gel filtration column, and to concentrate GlnKR47W before the G75 and hydroxylapatite columns. In both cases, the pooled peak fractions from the hydroxylapatite column were dialysed against storage buffer (Kamberov *et al*., 1994) and stored in aliquots at −80°C.

A second purified preparation of GlnK and purified GlnKY51N was provided by Dr Quan Sun of our laboratory. These were purified by the same method, except that the final hydroxylapatite column was replaced by a phenyl-Sepharose chromatography step. For this, ammonium sulphate was added to the pooled peak from the G75 column to 25% saturation. This sample was loaded directly onto a 40 ml phenyl-Sepharose column (Pharmacia) equilibrated in 50 mM Tris-Cl, pH 7.5, 1 mM EDTA, 100 mM KCl, 25% saturation ammonium sulphate, and the column was washed extensively with the same buffer. Under these conditions, GlnK binds quantitatively to the column. The column was eluted with a 250 ml gradient of ammonium sulphate from 25% to 0% saturation in the same buffer. GlnK and GlnKY51N eluted near the end of the gradient. The purified peak fractions from the phenyl-Sepharose column were pooled, dialysed against storage buffer and stored in aliquots at −80°C, as above.

The phenyl-Sepharose step described above was also shown to work well for the purification of PII (P. Jiang and A. Pioszak, unpublished data). This step is superior to the hydroxylapatite step described before (Kamberov *et al*., 1994), as hydroxylapatite chromatography required dialysis of the sample to remove salt that was present during the prior gel filtration chromatography step. Use of phenyl-Sepharose in place of hydroxylapatite eliminates the need for a slow dialysis step, while providing similar purification.

Protein concentrations were determined according to the method of Lowry *et al*. (1951).

Purified UTase/UR, PII, ATase, GS, NRI and NRII

Preparations of these proteins have been described previously (Jiang *et al*., 1997; Kamberov *et al*., 1994). Protein concentrations were determined according to the method of Lowry *et al*. (1951).

Preparation of PII∼UMP and GlnK∼UMP

To prepare these uridylylated proteins, 500 μl reaction mixtures containing 100 mM Tris-Cl, pH 7.5, 100 mM KCl, 25 mM $MgCl_2$, 500 μM ATP, 200 μM 2-ketoglutarate, 1 mM dithiothreitol (DTT), 120 μM GlnK or PII and 500 μM [α-^{32}P]-UTP were incubated at 37°C for 30 min. Glycerol and KCl were then added to 10% (v/v) and 350 mM final concentrations respectively. The reaction mixtures were then heated to 60°C for 15 min to inactivate the UTase/UR. Reaction mixtures were subjected to chromatography on 20 ml Sephadex-G50 columns in 50 mM Tris-Cl, pH 7.5, 10% glycerol, 1 mM DTT, 200 mM KCl to separate protein from unincorporated UTP. Peak fractions containing GlnK∼UMP or PII∼UMP were stored directly at −20°C.

Assays for the phosphorylation of NRI, adenylylation of GS, uridylylation of PII or GlnK and deuridylylation of PII∼UMP or GlnK∼UMP

These assays were carried out essentially as described

previously (Atkinson *et al*., 1994; Kamberov *et al*., 1994; Jiang *et al*., 1997) with modifications as noted in the figure legends. For the ATase, UTase and UR assays, initial rates were determined after fitting of progress curves using Enzfit, as described previously (Kamberov *et al*., 1995; Jiang *et al*., 1998a).

Acknowledgements

This work was supported by grant GM47460 from the NIH. We thank Dr Quan Son for providing a preparation of purified GlnK and the preparation of GlnKY51N used here, and Dr W. C. van Heeswijk for reviewing an earlier version of this paper.

References

Allikmets, R., Gerrard, B., Court, D., and Dean, M. (1993) Cloning and organization of the *abc* and *mdl* genes of *Escherichia coli*: relationship to eucaryotic multidrug resistance. *Gene* **136:** 231–236.

Atkinson, M.R., and Ninfa, A.J. (1998) Role of the GlnK signal transduction protein in the regulation of nitrogen assimilation in *Escherichia coli*. *Mol Microbiol* **29:** 431–447.

Atkinson, M.R., Kamberov, E.S., Weiss, R.L., and Ninfa, A.J. (1994) Reversible uridylylation of the *Escherichia coli* PII signal transduction protein regulates its ability to stimulate the dephosphorylation of the transcription factor nitrogen regulator I (NRI or NtrC). *J Biol Chem* **269:** 28288–28293.

Backman, K., Chen, Y.-M., and Magasanik, B. (1981) Physical and genetic characterization of the *glnA-glnG* region of the *Escherichia coli* chromosome. *Proc Natl Acad Sci USA* **78:** 3743–3747.

Brown, M.S., Segal, A., and Stadtman, E.R. (1971) Modulation of glutamine synthetase adenylylation and deadenylylation is mediated by metabolic transformation of the PII-regulatory protein. *Proc Natl Acad Sci USA* **68:** 2949–2953.

Bueno, R., Pahel, G., and Magasanik, B. (1985) Role of *glnB* and *glnD* gene products in the regulation of the *glnALG* operon of *Escherichia coli*. *J Bacteriol* **164:** 816–822.

Carr, P.D., Cheah, E., Suffolk, P.M., Vasudevan, S.G., Dixon, N.E., and Ollis, D.L. (1996) X-ray structure of the signal transduction protein PII from *Escherichia coli* at 1.9A. *Acta Cryst* **D52:** 93–104.

Engleman, E.G., and Francis, S.H. (1978) Cascade control of glutamine synthetase. II. Metabolic regulation of the enzymes in the cascade. *Arch Biochem Biophys* **191:** 602–619.

He, L., Soupene, E., and Kustu, S. (1997) NtrC is required for control of *Klebsiella pneumoniae* NifL activity. *J Bacteriol* **179:** 7446–7455.

He, L., Soupene, E., Ninfa, A.J., and Kustu, S. (1998) A physiological role for the GlnK protein: relief from NifL inhibition under nitrogen-limiting conditions. *J Bacteriol* **180:** 6661–6667.

van Heeswijk, W.C., Stegeman, B., Hoving, S., Molenaar, D., Kahn, D., and Westerhoff, H.V. (1995) An additional PII in *Escherichia coli*: a new regulatory protein in the glutamine synthetase cascade. *FEMS Microbiol Lett* **132:** 153–157.

van Heeswijk, W.C., Hoving, S., Molenaar, D., Stegeman, B., Kahn, D., and Westerhoff, H.V. (1996) An alternative PII protein in the regulation of glutamine synthetase in *Escherichia coli*. *Mol Microbiol* **21:** 133–146.

Jiang, P., Atkinson, M.R., Kamberov, E.S., Zucker, P., Schefke, B.R., Chandran, P., *et al*. (1997) Structure/function analysis of the PII signal transduction protein of *Escherichia coli*. Genetic separation of the interactions with receptors. *J Bacteriol* **179:** 4342–4353.

Jiang, P., Peliska, J.A., and Ninfa, A.J. (1998a) Enzymological characterization of the signal-transducing uridylyltransferase/uridylyl-removing enzyme (EC 2.7.7.59) of *Escherichia coli* and its interaction with the PII protein. *Biochemistry* **37:** 12782–12794.

Jiang, P., Peliska, J.A., and Ninfa, A.J. (1998b) Reconstitution of the signal-transduction bicyclic cascade responsible for the regulation of Ntr gene transcription in *Escherichia coli*. *Biochemistry* **37:** 12795–12801.

Jiang, P., Peliska, J.A., and Ninfa, A.J. (1998c) The regulation of *Escherichia coli* glutamine synthetase revisited: role of 2-ketoglutarate in the regulation of glutamine synthetase adenylylation state. *Biochemistry* **37:** 12802–12810.

Kamberov, E.S., Atkinson, M.R., Feng, J., Chandran, P., and Ninfa, A.J. (1994) Sensory components controlling bacterial nitrogen assimilation. *Cell Mol Biol Res* **40:** 175–191.

Kamberov, E.S., Atkinson, M.R., and Ninfa, A.J. (1995) The *Escherichia coli* PII signal transduction protein is activated upon binding 2-ketoglutarate and ATP. *J Biol Chem* **270:** 17797–17807.

Keener, J., and Kustu, S. (1988) Protein kinase and phosphoprotein phosphatase activities of nitrogen regulatory proteins NTRB and NTRC of enteric bacteria: roles of the conserved amino terminal domain of NTRC. *Proc Natl Acad Sci USA* **85:** 4976–4980.

Liu, J., and Magasanik, B. (1995) Activation of the dephosphorylation of nitrogen regulator I-phosphate of *Escherichia coli*. *J Bacteriol* **177:** 926–931.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L., and Randall, R.J. (1951) Protein measurement with Folin phenol reagent. *J Biol Chem* **193:** 265–275.

Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) *Molecular Cloning, a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, p. 68 and p. 250.

Ninfa, A.J., and Magasanik, B. (1986) Covalent modification of the *glnG* product, NR_I, by the *glnL* product, NR_{II}, regulates the transcription of the *glnALG* operon in *Escherichia coli*. *Proc Natl Acad Sci USA* **83:** 5909–5913.

Rhee, S.G., Chock, P.B., and Stadtman, E.R. (1989) Regulation of *Escherichia coli* glutamine synthetase. *Adv Enzymol (related areas Mol Biol)* **62:** 37–92.

Saiki, R.K. (1990) Amplification of genomic DNA. In *PCR Protocols, a Guide to Methods and Applications*. Innis, M.A., Gelfand, D.H., Sninsky, J.J., and White, T.J. (eds). San Diego, CA: Academic Press, pp. 13–20.

Schauder, B., Blocker, H., Frank, R., and McCarthy, J.E.G. (1987) Inducible expression vectors incorporating the

Escherichia coli atpE translation initiation region. *Gene* **52:** 279–283.

Senior, P.J. (1975) Regulation of nitrogen metabolism in *Escherichia coli* and *Klebsiella aerogenes*: studies with the continuous-culture technique. *J Bacteriol* **123:** 407–418.

Wong, P.-K., Popham, D., Keener, J., and Kustu, S. (1987) *In vitro* transcription of the nitrogen fixation regulatory operon nifLA of *Klebsiella pneumoniae*. *J Bacteriol* **169:** 2876–2880.

Xu, Y., Cheah, E., Carr, P.D., van Heeswijk, W.C., Westerhoff, H.V., Suhhash, V.G., and Ollis, D.L. (1998) GlnK, a PII-homologue: structure reveals ATP binding site and indicates how the T loops may be involved in molecular recognition. *J Mol Biol* **282:** 149–165.