doi:10.1016/j.jmb.2010.09.046 J. Mol. Biol. (2010) 404, 522–536

Contents lists available at www.sciencedirect.com

Journal of Molecular Biology

journal homepage: http://ees.elsevier.com.jmb

Reversible Adenylylation of Glutamine Synthetase Is Dynamically Counterbalanced during Steady-State Growth of *Escherichia coli*

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Received 18 May 2010; received in revised form 12 August 2010; accepted 19 September 2010
Available online 29 September 2010

Edited by I. B. Holland

Keywords:
2-oxoglutarate; covalent modification; glutamine; mathematical model; nitrogen assimilation

Glutamine synthetase (GS) is the central enzyme for nitrogen assimilation in *Escherichia coli* and is subject to reversible adenylylation (inactivation) by a bifunctional GS adenylyltransferase/adenylyl-removing enzyme (ATase). *In vitro*, both of the opposing activities of ATase are regulated by small effectors, most notably glutamine and 2-oxoglutarate. *In vivo*, adenylyltransferase (AT) activity is critical for growth adaptation when cells are shifted from nitrogen-limiting to nitrogen-excess conditions and a rapid decrease of GS activity by adenylylation is needed. Here, we show that the adenylyl-removing (AR) activity of ATase is required to counterbalance its AT activity during steady-state growth under both nitrogen-excess and nitrogen-limiting conditions. This conclusion was established by studying AR^-/AT^+ mutants, which surprisingly displayed steady-state growth defects in nitrogen-excess conditions due to excessive GS adenylylation. Moreover, GS was abnormally adenylylated in the AR^- mutants even under nitrogen-limiting conditions, whereas there was little GS adenylylation in wild-type strains. Despite the importance of AR activity, we establish that AT activity is significantly regulated *in vivo*, mainly by the cellular glutamine concentration. There is good general agreement between quantitative estimates of AT regulation *in vivo* and results derived from previous *in vitro* studies except at very low AT activities. We propose additional mechanisms for the low AT activities in vivo. The results suggest that dynamic counterbalance by reversible covalent modification may be a general strategy for controlling the activity of enzymes such as GS, whose physiological output allows adaptation to environmental fluctuations.

Introduction

In enteric bacteria, nitrogen is assimilated via two central nitrogen intermediates, glutamine (Gln) and glutamate (Glu). Their biosyntheses are elaborately controlled, depending on external environments. The regulations are through a wired circuit of reactions catalyzed by glutamine synthetase (GS,

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Abbreviations used: GS, glutamine synthetase; ATase, adenylyltransferase/adenylyl-removing enzyme; AT, adenylyltransferase; AR, adenylyl-removing; GOGAT, glutamate synthase; GDH, glutamate dehydrogenase; 2OG, 2-oxoglutarate.

encoded by *glnA*), glutamate synthase (GOGAT, encoded by *gltBD*), and glutamate dehydrogenase (GDH, encoded by *gdhA*).

$$\mathrm{Glu} + \mathrm{NH_3} + \mathrm{ATP} \xrightarrow{\mathrm{GS}} \mathrm{Gln} + \mathrm{ADP} + \mathrm{P_i}$$
$$\mathrm{2OG} + \mathrm{Gln} + \mathrm{NADPH} + \mathrm{H^+} \xrightarrow{\mathrm{GOGAT}} 2\,\mathrm{Glu} + \mathrm{NADP^+}$$
$$\mathrm{2OG} + \mathrm{NH_3} + \mathrm{NADPH} + \mathrm{H^+} \xrightarrow{\mathrm{GDH}} \mathrm{Glu} + \mathrm{NADP^+}$$

2-Oxoglutarate (2OG) serves as the carbon skeleton of central nitrogen metabolism. In an ATP-dependent manner, Glu can be synthesized by the combined action of GS and GOGAT. GDH catalyzes the alternative pathway for Glu synthesis.

A myriad of previous studies have established that GS occupies a central point of the regulation.[1] GS is composed of 12 identical subunits and is the only enzyme that catalyzes Gln synthesis. GS is regulated by several mechanisms, including (a) cumulative feedback inhibition by multiple end products of Gln metabolism, (b) repression and derepression of *glnA* transcription, and (c) reversible covalent modification of each subunit by adenylylation and deadenylylation of a specific tyrosine residue.

Adenylylation of GS leads to alteration of various catalytic properties, including inactivation of its biosynthetic activity in the presence of Mg^{2+}.[2–4] Both of the adenylylation and deadenylylation reactions require catalysis by a single but bifunctional adenylyltransferase/adenylyl-removing enzyme (ATase, encoded by *glnE*):[2,4–6]

$$\mathrm{GS} + \mathrm{ATP} \xrightarrow{\text{ATase (adenylyltransferase activity)}} \mathrm{GS{-}AMP} + \mathrm{PP_i}, \text{ and}$$
$$\mathrm{GS{-}AMP} + \mathrm{P_i} \xrightarrow{\text{ATase (adenylyl-removing activity)}} \mathrm{GS} + \mathrm{ADP}.$$

ATase is composed of two homologous halves, with the N-terminal half carrying adenylyl-removing (AR) activity and the C-terminal half carrying adenylyltransferase (AT) activity.[7,8] Extensive biochemical studies, pioneered by Stadtman and colleagues and later by other researchers, have demonstrated that ATase is regulated by several metabolites coupled with the regulatory protein P_{II} and its paralogue GlnK (Fig. S1).[4,5,9–15] *In vitro*, Gln acts directly on ATase and indirectly through P_{II} or GlnK to favor the adenylylation reaction. 2OG can exert qualitatively different effects depending on its concentrations. At a certain concentration range *in vitro*, it acts indirectly through P_{II} to favor the deadenylylation reaction. It has been shown that the combined action of adenylylation and deadenylylation by regulation of the metabolites determines the level of GS adenylylation *in vitro*[11,16–18] and in permeabilized cells.[19] The regulatory effect of Gln on GS adenylylation and activity is compatible with the general role of the Gln pool size as an internal indicator of external nitrogen availability.[20] However, the regulatory mechanisms established from *in vitro* studies do not necessarily all operate *in vivo*.[21] It is therefore critical to address what regulatory mechanisms operate *in vivo*. This demands quantitative approaches *in vivo*, preferably in combination with mathematical analyses. A recent metabolomics-driven quantitative study has revealed that both the Gln and the 2OG pool sizes change rapidly in response to sudden shifts of external nitrogen availability.[22] By an order of magnitude or more, their internal concentrations are the two most dramatically changed among many metabolites.

The physiological role of ATase as a whole enzyme has been long revealed in detail.[23,24] Cells grown under a nitrogen-limiting condition may encounter a sudden shift to a nitrogen-excess/sufficient condition. Without the enzyme (no AT activity), abundantly expressed GS cannot be inactivated immediately. This would lead to drainage of the Glu pool, resulting in a prolonged growth defect during the adaptation. However, unlike *in vitro* studies, *in vivo* dissecting the opposing activities of ATase is more challenging and the information is limited. Here we address the physiological role of the AR activity of ATase in *Escherichia coli*. We demonstrate that an imbalance of the AT and AR activities in AR^-/AT^+ mutants leads to excessive adenylylation of GS irrespective of external nitrogen sources. But the impact of such excessive adenylylation on steady-state cell growth depends on external nitrogen sources. The results suggest regulation of AT activity *in vivo*. We subsequently deduced the AT activities from measured data via a simple mathematical model. Correlation of the *in vivo* AT activities with the metabolite pool data revealed significant regulation by Gln, but insignificant by 2OG, for the range of concentrations observed for the AR^- mutants. This dependence is compared to the results derived from a detailed *in vitro* characterization of AT activity.[18] The quantitatively derived results are obtained by developing another mathematical model to capture the *in vitro* results. The *in vivo* and *in vitro* results agree well except for the regime with very low AT activities. Differences in the latter can be readily accounted for by either a basal AT activity at low Gln concentrations or an inhibitory effect on P_{II}-activated AT activity at high 2OG concentrations. Both scenarios are permissible given the available *in vitro* data. Our result on the dynamic counterbalance of AT/AR activities *in vivo* is reinforced by several previous observations on reversible covalent enzyme modifications. The result naturally suggests reasons why a removing functionality may be a general requirement for controlling enzyme modifications.

Results

Mutations in the AR domain of ATase suppress the growth defects of a GOGAT$^-$ mutant

In order to distinguish the physiological role of the two opposing functions of ATase, we employed two *glnE* alleles that specifically compromise the AR activity of ATase (AR$^-$). The first one, *glnE466*, was originally isolated as a mutation that suppresses the growth defect of a GOGAT$^-$ mutant on minimal agar plate containing 0.1 mM NH_4^+ as the sole nitrogen source.[25] The mutation by itself does not offer a selectable phenotype for genetic transfer. Two different linkage markers were utilized for cotransfer with *glnE466* in this report (*ΔyqiK744::kan* and *zgi-3602::cat*). This mutation was also found to suppress the growth defects of a *ΔgltD* mutant on minimal media with alternative sole nitrogen sources such as arginine (Fig. 1; comparing the *ΔgltD glnE466* strain FG1312 and the *ΔgltD glnE*$^+$ strain FG1294; the phenotype was used for genetic identifications; *glnE*$^+$ stands for the wild-type *glnE* allele). The mutation was characterized by sequencing the *glnE* coding region as a single nucleotide substitution that alters Lys317 to Thr. Lys317 is perfectly conserved in all ATase homologues thus far examined.[26] In the crystal structure of the N-terminal half of *E. coli* ATase, the side chain of Lys317 points into the AR catalytic center and is postulated to be involved in catalysis by interacting with the phosphates of ADP.[26] Therefore, we inferred *glnE466* as an AR loss-of-function mutation.

The second mutation carries two nucleotide substitutions that alter both Asp173 and Asp175 to Asn. The mutant enzyme ATase^{D173N,D175N} was first reported in an *in vitro* study.[27] It lacks the AR activity but retains significant AT activity that is synergistically activated by P_{II} and Gln. It possesses similar K_M values for both P_{II} and Gln to the wild-type ATase. We constructed a chromosomal version of this mutation and designated the allele as *glnE475*. In an identical fashion as for *glnE466*, *glnE475* suppressed the growth defects of the *ΔgltD* mutant on minimal media containing arginine (Fig. 1; comparing the *ΔgltD glnE475* strain FG1438 to FG1312 and FG1294).

Loss of AR activity has a different physiological effect from loss of AT activity

During the characterization of *glnE466* mutation, we noticed that it caused a reversed phenotype in a GOGAT$^+$ background: a mild but reproducible growth defect when growing in high-NH_4^+ medium. We then compared its effect on steady-state growth with other *glnE* mutations, including the biochemically characterized *glnE475*;[27] a deletion mutation, *ΔglnE*, from the Keio deletion collection;[28] an insertion mutation, *glnE::*Tn*5*-KAN-I-SceI, from the Blattner knockout collection;[29] and another early reported insertion mutation, *glnE::*Tn*5-131*.[30] With 10 mM NH_4^+, both the *glnE*$^+$ control strains FG1301 and FG1407 (*ΔyqiK::kan*) showed an identical doubling time of 63 min (Fig. 2a). However, *glnE466* and *glnE475* mutants (FG1328 and FG1437, both with *ΔyqiK::kan*) displayed a mild growth defect, with measured doubling times of 74 and 77 min, respectively. None of the other *glnE* deletion or insertion mutants (FG1320, FG1413, and FG1411) displayed any steady-state growth defect.

It has been well documented that ATase (AT activity) is required for fast adaptation to a sudden increase in external nitrogen availability in *Salmonella enterica* serovar Typhimurium.[23,24] We examined the response of the various *E. coli glnE* mutants, preadapted to a nitrogen-limiting condition, upon an NH_4^+ upshift (Fig. 2b). Proline is a poor nitrogen

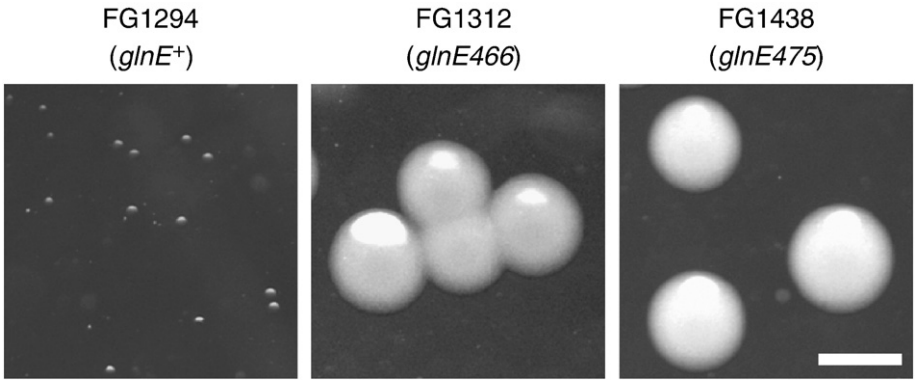

Fig. 1. AR$^-$ mutations suppress the growth defect of a *ΔgltD* mutant with arginine as the sole nitrogen source. Colonies of FG1294 (*ΔgltD ΔyqiK::kan*), FG1312 (*ΔgltD glnE466 ΔyqiK::kan*), and FG1438 (*ΔgltD glnE475 ΔyqiK::kan*) were grown on agar medium for 4 days. The scale bar represents 0.5 mm.

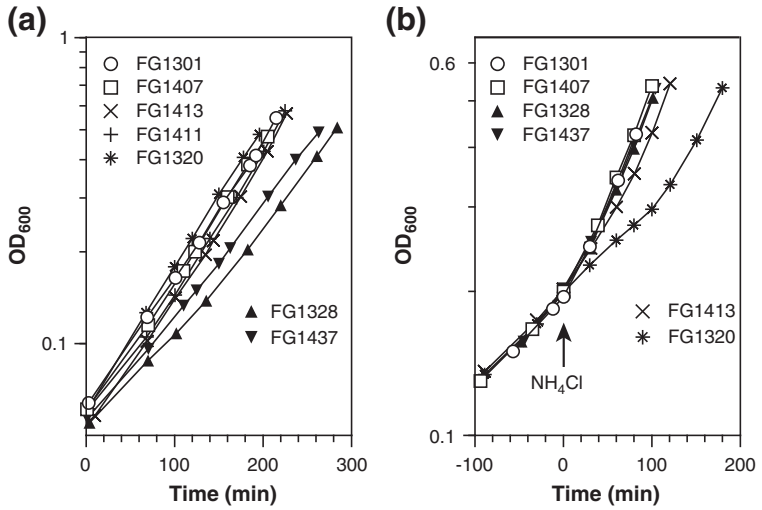

Fig. 2. Physiological effects of *glnE* mutations. (a) Batch culture growth in 10 mM NH_4^+. Doubling times (in minutes) are 63 ± 1 (FG1301, *glnE*$^+$), 63 ± 0 (FG1407, *glnE*$^+$ *ΔyqiK::kan*), 74 ± 2 (FG1328, *glnE466 ΔyqiK::kan*), 77 ± 0 (FG1437, *glnE475 ΔyqiK::kan*), 65 ± 2 (FG1413, *glnE::*Tn*5*-KAN-I-SceI), 64 ± 2 (FG1411, *glnE::*Tn*5-131*), and 64 ± 2 (FG1320, *ΔglnE*). (b) Response to an NH_4^+ upshift. Cells were preadapted in medium with 10 mM proline as the sole nitrogen source. The upshift was performed by adding NH_4^+ to a final concentration of 40 mM at time zero.

source. Upon an NH_4^+ upshift from proline growth, the *glnE*$^+$ strains FG1301 and FG1407 adapted to a faster growth rate after ~30 min. Consistent with the previous observation in *S. enterica*, the *glnE* deletion strain FG1320 displayed a sizable growth delay after the NH_4^+ upshift. Growth delay was also observed when other poor nitrogen sources such as arginine or threonine were used in the preshift cultures. The *glnE::*Tn*5*-KAN-I-SceI strain FG1413, however, showed only a marginal growth delay, suggesting that the insertion does not significantly affect the AT activity of the C-terminal half of ATase. In the *glnE466* and *glnE475* strains (FG1328 and FG1437), no growth delay was observed after the NH_4^+ upshift, indicating their retention of significant AT activities. The combined observations in both the steady-state growth and the upshift response demonstrate that AR activity is required in a different physiological situation from AT activity.

As AT activity is critical during growth adaptation to a sudden increase in external nitrogen availability, we also tested the possible role of the opposing AR activity in a completely reversed situation: a sudden decrease in external nitrogen availability termed as a nitrogen downshift. The downshift was achieved by stripping of cell cultures, preadapted in proline plus NH_4^+ medium, free of NH_4^+ through a fast filtration process. At our experimental resolution where growth adaptation could be monitored starting at as early as 75 s postshift, no significant difference in postshift growth was observed between the *glnE*$^+$ and the AR$^-$ strains (Fig. S2).

Excessive GS adenylylation in AR$^-$ mutants causes internal Gln limitation

To explore the cause of growth defect of AR$^-$ mutants in NH_4^+ medium, we first examined their Glu and Gln pools together with other *glnE* mutants. For the Glu pools, there was little difference among any of the mutants and its congenic *glnE*$^+$ strains (Fig. 3a). However, the Gln pools varied depending on the mutations† (Fig. 3b). In both AR$^-$ mutants (*glnE466* FG1468 and *glnE475* FG1469, both linked with *zgi-3063::*FRT), the values were approximately half of the *glnE*$^+$ strains (FG1301 and FG1449; the latter linked with *zgi-3063::*FRT).

Next, we quantified *glnA* expression level by using a reporter (Fig. 3c; gray bars). The above strains for Glu and Gln pool determination contain a chromosomal *glnA-lacZ* fusion located at the *lac* locus. Derepression of *glnA* transcription is a key indication of external nitrogen limitation and/or internal Gln limitation. For the AR$^-$ mutants in NH_4^+ medium, the *glnA* expression increased more than 10-fold that of the *glnE*$^+$ strains. The levels of *glnA* expression in the other three deletion or insertion *glnE* mutants were more or less in the repressed regime.

We then measured the GS adenylylation state of cells grown in NH_4^+ medium (Fig. 3c; black bars). As expected, no GS adenylylation was observed in the *ΔglnE* strain (FG1320) where the *glnE* coding region is completely eliminated.[28] The two *glnE* insertion mutants (FG1413 and FG1411) showed different but significant GS adenylylation, indicating the insertions do not completely abolish the AT activity of ATase. In the control *glnE*$^+$ strains (FG1301 and FG1449), up to half of GS subunits were adenylylated, with the average number of adenylylated GS

†In mutants *ΔglnE* (FG1320), *glnE*::Tn*5*-KAN-I-SceI (FG1413), and *glnE*::Tn*5-151* (FG1411), the Gln pools were similar to those in the *glnE*$^+$ strains at OD_{600} ~0.2 but increased (up to ~2-fold) after one doubling (Fig. 3b). How this phenomenon is related to the AT activity, which is either eliminated or disturbed in these three mutants, is unclear.

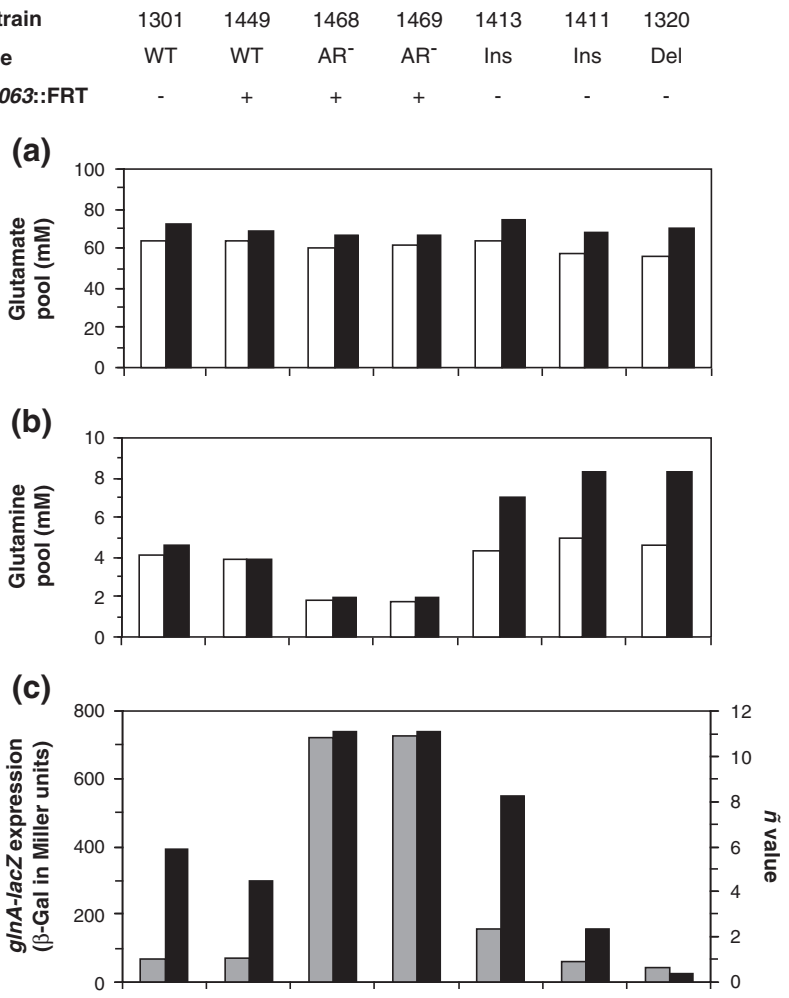

Fig. 3. Characterization of *glnE* mutants during steady-state growth in NH_4^+. All experiments were performed at least twice, with the data shown in average. (a) Glu and (b) Gln pool sizes. Samples were taken at OD_{600} ~0.2 (white) and ~0.4 (black). (c) *glnA* expression (gray) and GS adenylylation state (black).

subunits per GS dodecamer complex, $\tilde{n}$, being 5.9 and 4.5. In the AR^- mutants (FG1468 and FG1469), however, GS was excessively adenylylated, with $\tilde{n}$ values being 11.1 in both strains. These results suggest that in wild-type cells with NH_4^+ as the nitrogen source, AR activity is required to prevent excessive GS adenylylation by AT activity, that is, a dynamic balance between the AT and the AR activities keeps GS adenylylation at midlevel. At the same time, cells are able to maintain a sufficient internal Gln concentration for an optimal growth. Losing the counterbalance, the AR^- mutants are internally limited on Gln even when using the preferred nitrogen source NH_4^+.

Lack of AR activity perturbs the GS adenylylation state under nitrogen-limiting conditions without causing a growth defect

We further characterized the growth of the *glnE* mutants with other nitrogen sources. When cytidine, alanine, or serine was used as the sole nitrogen source, the $glnE^+$ control strain FG1301 grew at almost the same growth rate as in NH_4^+ medium (Fig. 4a). Their measured Gln pool sizes are no less than that in NH_4^+, and the *glnA* expression levels are no more than 3-fold higher than that in NH_4^+. These three nitrogen sources, as well as NH_4^+, can be defined as nitrogen-excess/sufficient conditions. By contrast, when aspartate, proline, glycine, or arginine was used as the sole nitrogen source, FG1301 showed slower growth to different degrees (Fig. 4a). In these cases, the Gln pools are no more than 20% of that in NH_4^+, and the *glnA* expression levels were elevated more than 10-fold of that in NH_4^+. These four nitrogen sources therefore provide cells with limited nitrogen and are referred to as nitrogen-limiting conditions (or poor nitrogen sources). Our observation in *E. coli* is in good agreement with what has been demonstrated in *S. enterica* and *Klebsiella pneumoniae*.[20,31]

We compared the growth rates of *glnE* mutants with $glnE^+$ strains in the eight nitrogen sources (four nitrogen-excess and four nitrogen-limiting conditions). The *ΔglnE* strain (FG1320) showed almost no growth phenotype (Fig. 4b). This result

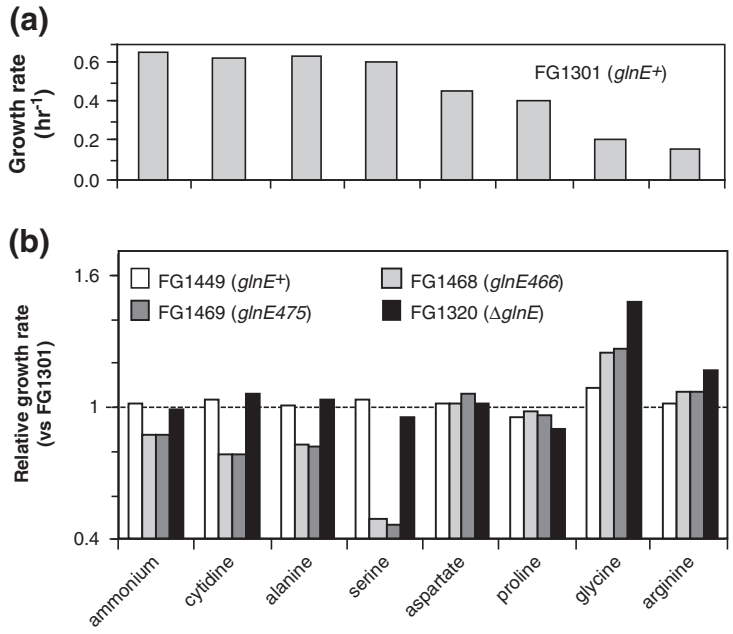

Fig. 4. Growth of *glnE* mutants in various nitrogen sources. All cultures were repeated at least twice, with the growth rate shown in average. (a) Growth rates of the $glnE^+$ control strain FG1301. (b) Relative growth rates of FG1449 ($glnE^+$ *zgi-3603*::FRT), FG1468 (*glnE466 zgi-3603*::FRT), FG1469 (*glnE475 zgi-3603*::FRT), and FG1320 (*ΔglnE*), compared to that of FG1301 in (a).

was expected, as it has been reported that ATase is dispensable during steady-state growth regardless of external nitrogen availability.[24] Strikingly, the AR^- mutants (FG1468 and FG1469) displayed growth defects in all of the four nitrogen-excess conditions, but not in any of the four nitrogen-limiting conditions. AR activity therefore appeared to be required only for optimal growth under nitrogen-excess conditions.

The lack of any growth defect for the AR^- mutants in the nitrogen-limiting conditions could be explained if AT activity were tightly repressed to nonexistence where AR activity was no longer required. We therefore examined the adenylylation state of GS in cells grown with proline as the sole nitrogen source (Fig. 5; black bars). As expected for a nitrogen-limiting condition, the $glnE^+$ strains (FG1301 and FG1449) showed little GS adenylylation. To our surprise, the AR^- strains (FG1468 and FG1469) displayed GS adenylylation levels significantly higher than those of the control strains, with $\tilde{n}$ values of 7.2 and 7.3, respectively. This observation suggests that even in nitrogen-limiting conditions, AT has a significant activity that requires being counterbalanced by AR activity. This counterbalance allows the wild-type cells to maintain a low adenylylation state of GS. Interestingly, all strains showed a similar, fully derepressed *glnA* expression level (Fig. 5; gray bars). The higher GS adenylylation in the AR^- mutants therefore does not trigger a higher level of gene expression from the *glnA* promoter than that in the wild type.

Without further elevated *glnA* expression under the nitrogen-limiting conditions, the abnormal GS adenylylation by loss of AR activity translates into less active GS amount. If the already low internal Gln level were the result of a limitation on active GS amount, this would lead to a further decrease in the pool size, similar to the case in NH_4^+ medium. However, we found that the Gln pools were almost

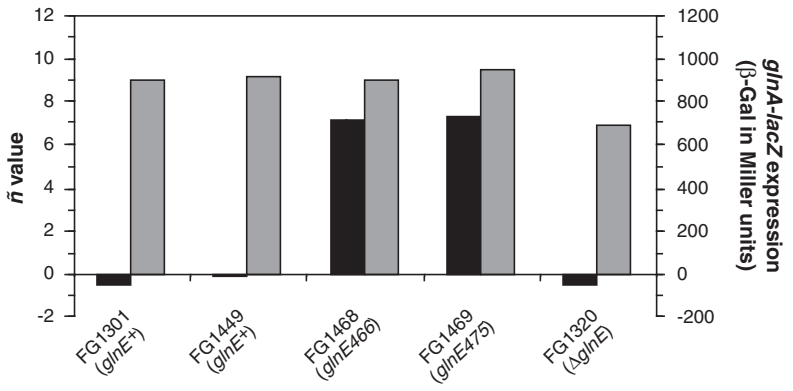

Fig. 5. GS adenylylation state and *glnA* expression of AR^- mutants during steady-state growth in proline. Black, GS adenylylation state; gray, *glnA* expression. All experiments were performed at least twice, with data shown in average. Strains are the same as in Fig. 4.

identical between the AR$^-$ mutants and the *glnE*$^+$ strains grown in proline and in other poor nitrogen sources (Table S3). These results, rather surprising in their appearance, led us to hypothesize that the internal NH_4^+ substrate, rather than the active enzyme amount, is the limitation under the nitrogen-limiting conditions (see Discussion). Nevertheless, the result is consistent with the previous demonstration in *S. enterica* that growth rates are correlated with the Gln pool sizes according to nitrogen availability.[20]

Analysis

As shown above, the $\tilde{n}$ values of the AR$^-$ cells from proline culture are smaller than those from NH_4^+ growth (Figs. 3c and 5), implying *in vivo* regulation of AT activity depending on the different nitrogen sources. Quantitatively, the fact that GS is not completely adenylylated in the absence of AR may be another surprise, since any nonzero AT activity would eventually make all GS adenylylated. A simple way to rationalize the limited degree of GS adenylylation observed is the balance between AT activity and dilution effect due to newly synthesized (unadenylylated) GS. Adopting this view, we developed a simple mathematical model to deduce quantitatively the AT activity of the AR$^-$ mutants *in vivo*. We then correlated the results with measured metabolite pool data. These allowed us to quantitatively characterize the regulation of AT activity *in vivo* and compare the regulation to that characterized *in vitro*.

Quantitation of the *in vivo* AT activity

The adenylylation status of GS reflects the balance among adenylylation, deadenylylation, and dilution due to cellular growth. Adenylylation and deadenylylation is governed by the reaction scheme depicted in Fig. S3. The time dependence of the concentrations of adenylylated and unadenylylated GS subunits (denoted by x_a and x_u, respectively; a dot above the symbol denotes time derivative) is given by

$$\dot{x}_a = \kappa_t e_t x_u / (K_u + x_u) - \kappa_r e_t x_a / (K_a + x_a) - \lambda x_a \quad (1)$$

$$\dot{x}_u = \kappa_r e_t x_a / (K_a + x_a) - \kappa_t e_t x_u / (K_u + x_u) - \lambda x_u + \gamma \quad (2)$$

Here, e_t is the concentration of ATase, which has affinities K_a and K_u for the adenylylated and unadenylylated GS subunits, respectively. Adenylylation occurs at rate κ_t, and deadenylylation at rate κ_r. The latter is taken to be zero for the AR$^-$ mutant. The last term in Eq. (1) describes the dilution due to cellular growth at growth rate λ. γ denotes the rate of GS synthesis, which, in the absence of significant degradation, is just λx_t. $x_t \equiv x_a + x_u$ is the concentration of all GS subunits.

In the following, we will quantify AT activity by $k_t \equiv \kappa_t e_t / K_u$, which characterizes the rate of adenylylating all GS subunits. In steady state ($dx_a/dt = dx_u/dt = 0$), we obtain

$$k_t = \frac{\tilde{n}\lambda}{12 - \tilde{n}} + \frac{\tilde{n}\lambda}{12}\frac{x_t}{K_u} \quad (3)$$

where the adenylylation number $\tilde{n} \equiv 12 x_a / (x_u + x_a)$, the total GS amount x_t, and the growth rate λ are all measurable quantities. The *in vivo* value of K_u is uncertain. In the following, we apply one reported *in vitro* value, $K_u = 35$ μM.[18] Another available but much larger K_u, obtained under a very different condition,[17] has only limited effect on k_t.

Equation (3) was used to extract the AT activities k_t from the measured values of $\tilde{n}$, x_t, and λ for the AR$^-$ mutants grown with various nitrogen sources (see Table 1 and Table S4). The AT activities obtained were much higher in the nitrogen-excess than the nitrogen-limiting conditions. (Note that k_t values showed ~20-fold difference, while the adenylylation number $\tilde{n}$ only varied between ~7 and ~11.) The results indicate that AT activity is significantly regulated in the AR$^-$ mutants according to nitrogen availability.

In vivo regulation of AT activity

Both AT and AR activities are known to be regulated *in vitro*, directly by Gln and indirectly by both Gln and 2OG through P_{II} (Fig. S1). To determine the *in vivo* contribution of these two regulatory metabolites to AT activity, we measured the Gln and 2OG pool sizes for *glnE475* strain (FG1469) grown in various nitrogen sources (Table 1). A clear positive relationship emerged in a log–log plot of k_t against the Gln pool sizes, but not against the 2OG pool sizes (Fig. 6). These results indicate that within the observed ranges of internal concentrations, Gln is a dominant regulator of AT activity and acts in an almost linear fashion. However, the effect of 2OG does not become evident from this analysis.

Comparison to *in vitro* regulation of AT activity

The dependence of ATase activity on the concentrations of P_{II} and Gln has recently been characterized *in vitro* by Jiang *et al.*[18] To shed further light on the regulatory effects of Gln and 2OG, we compared the *in vivo* AT activity (k_t) deduced above with the reported *in vitro* results. To do so, we combined the results of Jiang *et al.* with those of an earlier *in vitro*

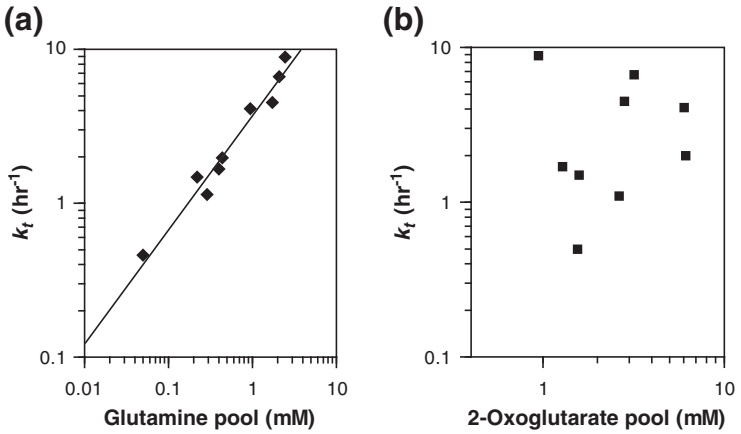

Fig. 6. AT activity as a function of metabolite pool sizes. *In vivo* AT activities k_t (listed in Table 1) are plotted in a log–log scale against (a) Gln and (b) 2OG pool sizes. The line represents a linear dependence of k_t on the Gln pool sizes.

study on the dependence of P_{II} urydylylation status on Gln and 2OG.[11] We then constructed a series of quantitative models describing the net effect of Gln and 2OG on the AT activity *in vitro* (see SI Analysis for details). One obstacle preventing a direct comparison of the results of this *in vitro* model with the *in vivo* derived AT activity is that most of the *in vitro* results were obtained at a fixed 2OG concentration of 0.05 mM.[18] This is significantly below the observed range of 2OG pool concentration for both the *glnE*$^+$ and the AR$^-$ strains. In the following, we assumed the simplest scenario whereby the *in vitro* values of the biochemical parameters describing the Gln–P_{II}–ATase interaction are the same at physiological 2OG concentrations. There may of course be additional 2OG-dependent interactions at the (much higher) *in vivo* 2OG levels. We explored the possibility that P_{II}-activated AT activity is inhibited by 2OG. Such an inhibition was documented in early *in vitro* studies[12,13] and later characterized at fixed Gln and P_{II} levels.[11]

In the *in vitro* models, the adenylylation flux is given by $\kappa_t^* e_t^* x_u/(K_u + x_u)$, where e_t^* is the *in vitro* ATase concentration, and the *in vitro* rate κ_t^* is a function of the Gln and 2OG concentrations [see Eqs. (S4) and (S6) in SI Analysis]. Using the models, we calculated the *in vitro* version of the rate k_t deduced above, $\kappa_t^* = \kappa_t^* e_t^* / K_u$, for the measured *in vivo* Gln and 2OG concentrations (Table S5). We assumed a one-to-one correspondence between the *in vitro* and the *in vivo* concentrations. The results are compared to the *in vivo* k_t shown in Table 1 (red symbols in Fig. 7). In each comparison, e_t^* is the only parameter adjusted to minimize the differences between k_t^* and k_t (weighted by the uncertainty in k_t). Values of the best-fit parameter e_t^{fit} are given for each model in Table S5. Good agreements between the *in vitro* and the *in vivo* results are seen for all but the condition with the lowest AT activity, corresponding to the lowest Gln concentration. For that condition, agreement is improved for the model with 2OG inhibition (red triangle in Fig. 7; perfect agreement is indicated by the black line).

The higher *in vivo* k_t value at the lowest Gln concentration could alternatively be accounted for by a basal AT activity. Such a basal AT activity has not been included in the models described above but are not excluded by the reported *in vitro* data,[18] since AT activity has not been characterized systematically in the regime of low Gln concentrations. We

Table 1. Measured data and deduced AT activities of *glnE475* mutant grown in different nitrogen sources

Nitrogen source[a]	Growth rate (h^{-1})	$\tilde{n}$	Total GS subunit (μM)	k_t (h^{-1})	Gln pool (mM)	2OG pool (mM)
Ammonium	0.60 ± 0.01	11.1 ± 0.0	103 ± 3	8.9 ± 1.3	2.45 ± 0.14	0.94 ± 0.07[b]
Cytidine	0.51 ± 0.01	11.0 ± 0.0	90 ± 3	6.7 ± 0.5	2.09 ± 0.02	3.18 ± 0.06
Alanine	0.50 ± 0.00	10.3 ± 0.0	89 ± 5	4.1 ± 0.4	0.95 ± 0.01	6.00 ± 0.43
Gly+Arg	0.50 ± 0.00	10.4 ± 0.0	92 ± 4	4.5 ± 0.3	1.73 ± 0.09	2.81 ± 0.14
Aspartate	0.47 ± 0.01	7.6 ± 0.1	104 ± 4	1.7 ± 0.2	0.40 ± 0.15	1.28 ± 0.03
Proline	0.39 ± 0.00	7.3 ± 0.1	129 ± 1	1.5 ± 0.1	0.22 ± 0.03	1.58 ± 0.26
Glycine	0.24 ± 0.00	10.3 ± 0.1	94 ± 5	2.0 ± 0.5	0.44 ± 0.03	6.12 ± 0.26
Arginine	0.18 ± 0.01	9.6 ± 0.2	104 ± 3	1.1 ± 0.3	0.29 ± 0.01	2.63 ± 0.04
Glutamate	0.12 ± 0.00	7.6 ± 0.0	119 ± 1	0.5 ± 0.0	0.05 ± 0.01	1.55 ± 0.09

[a] Strain FG1469 was grown with various sole nitrogen sources (10 mM total N). Gly + Arg, 5 mM glycine plus 1.25 mM arginine.
[b] For the *glnE*$^+$ strain FG1301, 2OG pool is ~0.5 mM.

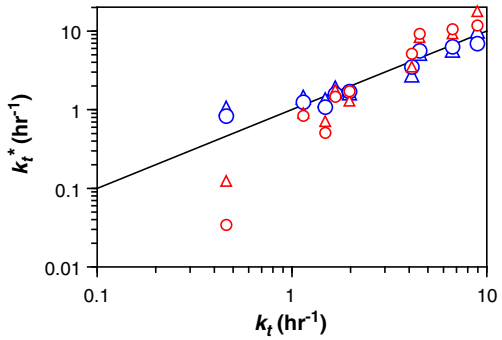

Fig. 7. Correlation of AT activities between calculated k_t^* from *in vitro* models and k_t deduced from *in vivo* measurement. Data are from Table 1 and Table S5. k_t^* values are from four different *in vitro* models: red circle, without 2OG inhibition and $\kappa_{t,bas}^*$; red triangle, with 2OG inhibition but without $\kappa_{t,bas}^*$; blue circle, without 2OG inhibition but with $\kappa_{t,bas}^*$; and blue triangle, with 2OG inhibition and $\kappa_{t,bas}^*$. The line represents the perfect agreement.

therefore repeated the analysis by including a basal activity in the *in vitro* models with and without the 2OG inhibition. The new models [Eqs. (S7) and (S8) in SI Analysis] now have two free parameters, e_t^* and a basal adenylylation rate $\kappa_{t,bas}^*$. Both are adjusted to minimize the differences between k_t^* and k_t (see Table S5). Figure 7 (blue symbols) shows the comparison for the two variants of the models using the best-fit basal activity $\kappa_{t,bas}^{fit}$. The model results are seen to agree well with the *in vivo* results with or without the 2OG inhibition. Both models with the 2OG inhibition and basal activity generate AT activities that show a linear dependence on the Gln concentrations as observed in Fig. 6a. However, the mechanisms are rather different, as explained in SI Analysis (Fig. S6).

Discussion

Physiological role of AR function during steady-state growth

The unexpected growth phenotypes caused by *glnE466* prompted us to conduct phenotypic comparisons among several *glnE* mutants. In comparison to *glnE475*, whose equivalent mutant enzyme was previously characterized *in vitro*,[27] all *glnE466* examinations yielded nearly identical outputs. This provides confirmation for the initial structure-based assumption of its loss of AR function. The two AR$^-$ mutations are genetically different but functionally indistinguishable. Among the other supposed *glnE*$^-$ mutations, only the deletion mutation *ΔglnE*[28] results in a total loss of AT activity. The insertion mutations *glnE*::Tn*5*-KAN-I-SceI[29] and *glnE*::Tn*5*-*151*[30] do not completely abolish AT function. Their resulting phenotypes are complex, probably due to remnants of truncated yet functional AT and, possibly, AR modules.

We examined physiological responses of various strains in four scenarios: the steady-state growth in nitrogen-excess and -limiting conditions, and the growth adaptation upon an NH_4^+ upshift and downshift. Because protein modification is a faster response compared to gene expression, it is logical that ATase is constitutively expressed[32] and present at all times preparing for nitrogen fluctuation. A phenotype by the loss of AT function during growth adaptation can be rationalized and has been experimentally explained.[23,24] Although the lack of significant adaptation phenotype by the loss of AR function upon a nitrogen downshift is somewhat surprising, it does not diminish the necessity for AR activity. In steady state, the allosterically regulated AT function may cause a problem if it possesses an unchecked residual activity. Indeed, the AR$^-$ mutants showed steady-state growth defects in nitrogen-excess conditions (Figs. 2a and 4). This is just the opposite of the adaptation but no steady-state phenotype by the loss of AT activity, suggesting a distinct counterbalancing role by the AR activity of ATase.

In NH_4^+ medium, the AR$^-$ mutants exhibited decreased Gln pools and elevated *glnA* expression (Fig. 3). They are internally limited in Gln due to the unbalanced inactivation of GS by AT activity. We conclude that in wild type, AR activity is required to prevent excessive adenylylation, thus allowing sufficient Gln synthesis for optimal growth under nitrogen-excess conditions. Under nitrogen-limiting conditions (e.g., in proline medium), the balancing act between AT and AR activities still occurs. However, the loss of AR function did not translate into further decreases of the already low Gln pool and growth rate under such conditions (Table S3 and Fig. 4). Serving as our current working hypothesis, one possible explanation is that the poor nitrogen sources could only supply limited NH_4^+ substrate internally through deamination(s), and cells synthesize excess GS to saturate the substrate for Gln synthesis. The GS subunit concentration under such conditions is ~100 μM. Determination of the internal NH_4^+ concentration is difficult. As 20 μM external NH_4^+ may still support a growth almost as fast as 10 mM NH_4^+, internal NH_4^+ concentration of cells using a poor nitrogen source could be much less than 20 μM. Therefore, a partial inactivation of GS in the AR$^-$ mutants (see $\tilde{n}$ values in Table 1 and Table S4) may still leave enough active enzymes to saturate the trace amount of internal NH_4^+ in concentrations and synthesize Gln at the same rate as in the wild type.

glnE466 was isolated as a mutation that suppresses the growth defect of a GOGAT$^-$ mutant grown with trace amount of NH_4^+.[25] In the absence of GOGAT, Glu biosynthesis can only be achieved through the action of GDH. Unlike GOGAT, GDH has a low affinity for NH_4^+. A suboptimal Glu pool size is the direct consequence of GOGAT$^-$ mutants grown in low NH_4^+ conditions and correlates with the growth defect.[23,33] Another characteristic of the GOGAT$^-$ mutants is their abnormally high Gln pool. Previously, we identified two types of suppressor mutation arisen from GOGAT$^-$ mutants.[25] One type of mutation overexpresses GDH, thus enhancing Glu biosynthesis even under low NH_4^+ conditions. The other is a *glnA* mutation that produces a partially impaired GS. The crippled enzyme restricts GS flux, resulting in a drop of the Gln pool together with a backup of the Glu pool in a GOGAT$^-$ background. The *glnA* mutation also shows a lower than normal Gln pool and a growth defect in a GOGAT$^+$ background. The latter two phenotypes are identical to what we described above for the AR$^-$ mutants. We also observed that the high Gln pool in the GOGAT$^-$ background is lowered by the AR$^-$ mutations, and the suboptimal Glu pool slightly increases under low NH_4^+ conditions. This suggests a similar mechanism of growth suppression by the *glnA* and AR$^-$ mutations, that is, a backup of the Glu pool caused by a limitation of GS flux. The only difference is that the former impairs GS by a structural mutation, while the latter inactivates the wild-type enzyme by excessive adenylylation.

Counterbalancing the dynamic regulation by a removing activity on reversible covalent modification

The AR$^-$ mutants enabled us to dissect the AR function from ATase and show its requirement for maintaining a proper GS adenylylation state during steady-state growth in both nitrogen-excess and -limiting conditions (Figs. 3c and 5). Such a requirement has been inferred as a probable cause for some previous *in vivo* observations.[34,35] In those cases, mutants impaired of regulatory components upstream of ATase also showed abnormal GS adenylylation in some nitrogen sources. Our result directly demonstrated that the proper GS adenylylation state during steady-state growth is achieved through a dynamic balance between AT and AR activities of ATase irrespective of external nitrogen sources. We firmly ruled out the possibility that the proper GS adenylylation state was achieved by merely regulated AT activity and an opposing dilution effect due to cell growth (an unlikely scenario that could be rationalized nonetheless under certain conditions). However, the dynamic balance itself is not a necessity in sustaining the maximal steady-state growth rate: for example, the strain lacking ATase altogether (FG1320) showed no growth defect in all nitrogen sources examined (Fig. 4b).

Theoretical analyses of model systems have shown that interconvertible enzyme cascades could achieve remarkable regulatory capabilities through dynamic balance such as signal integration, signal amplification, and sensitivity amplification.[36,37] Although such capacities were examined both *in vitro* and *in situ*,[17,19,38] *in vivo* proof has been difficult to obtain due to the bifunctionality of the enzymes. In addition, the effector concentrations applied *in vitro* and *in situ* do not necessarily represent the physiological ranges of the signals. The issue can be addressed only by *in vivo* studies. Our combined genetic and metabolic efforts provide *in vivo* demonstration of dynamic balance for the reversible modification of GS. The result suggests that this system has been selected to attain signal integration through its regulated metabolic network. The property of signal integration could act as a mechanism for cells to respond to nutrient fluctuations that require rapid up- or down-regulation of GS activity. This proposition needs to be further examined quantitatively *in vivo*. Regardless, our data show plainly that even proper regulation of the steady-state adenylylation status requires a dynamic balance. Without AR activity, we see that a ~20-fold decrease in AT activity results in only ~30% drop in the degree of adenylylation ($\tilde{n}$; Table 1). Alternatively, a 10-fold drop in $\tilde{n}$ would require a much more dramatic decrease in k_t according to Eq. (3). This appears to be a rather general problem for unidirectional control of covalent modification, given the modification is relatively stable. Below, we mention additional steady-state instances of similar *in vivo* phenomena reported in the literature. Collectively, they suggest that the counterbalancing scheme may be a general feature for enzymes catalyzing reversible covalent modification of proteins whose primary physiological output is for rapid adaptation to environmental signals.

In the control circuit of *glnA* expression responding to nitrogen availability, the transcriptional activator NtrC is subject to reversible phosphorylation. Unlike GS, the modified, phosphorylated NtrC is the active form, activating the major *glnA* promoter under nitrogen-limiting conditions.[39] Its phosphorylation is achieved by the kinase function of the bifunctional NtrB, or through autophosphorylation by small phosphor donors such as acetyl phosphate.[40–42] Similar to the AR activity of ATase as to GS, the phosphatase function of NtrB asserts a dominant regulatory role determining the phosphorylation state of NtrC. Breaking the balance of the opposing activities by mutated NtrB with impaired phosphatase function, keeps NtrC in the phosphorylated form, resulting in high *glnA*

expression even under nitrogen-excess conditions.[43] Another example with resemblance to the reversible GS adenylylation control is the regulation of nitrogenase activity in the photosynthetic bacterium *Rhodospirillum rubrum*. It involves a reversible inactivation of dinitrogenase reductase by ADP ribosylation in response to the availability of fixed nitrogen and/or light level.[44] In this case, the opposing activities reside in two enzymes: DraT for ADP ribosylation and DraG for the removal. Both are constitutively expressed, with activities regulated involving P_{II} analogous to ATase.[45] In an analogy to our results here, one original record showed that a portion of dinitrogenase reductase is in the ADP-ribosylated, inactive form in a *draT*$^+$/*draG*$^-$ mutant under a N_2-fixing condition, but none was modified in the wild type‡.[46] The above observations together with the results of this study point to a common theme. In all cases, the removing activity of covalent protein modification is actively used to counterbalance the modifying activity even under conditions where such a modification appears not warranted in the first place.

Mathematical model of the modifying activity on GS adenylylation regulated by metabolites

The importance of AR activity on the dynamic balance does not imply that AT activity is not regulated. We deduced the AT activity in the AR$^-$ mutants from a simple mathematical model. The model describes the balance between adenylylation by the ATase and dilution due to newly synthesized (unadenylylated) GS during growth. The result reveals regulation of AT activity *in vivo*, significantly by the Gln pool, but insignificantly by the 2OG pool (Fig. 6). Overall, the pattern of *in vivo* regulation of AT activity obtained is in good agreement with predictions of models constructed on the basis of *in vitro* studies of AT activity (Fig. 7). Differences between the *in vivo* and the *in vitro* results at very low AT activities may be attributed to a lack of *in vitro* data at the physiologically relevant regimes of metabolite concentrations, that is, very low Gln concentrations (0.1 mM and below) and high 2OG concentrations (1–10 mM). The difference can be accounted for by either an inhibitory effect of 2OG on the activation of AT activity by P_{II} or a basal AT activity at very low Gln concentrations. In addition, highly expressed GlnK in AR$^-$ mutants due to the internally limited Gln could also contribute to the disagreement. The involvement of GlnK is uncertain, since stimulation of AT activity by GlnK *in vitro* is known to be much less efficient than P_{II} stimulation.[14] Further *in vitro* experiments are required to characterize the existence and magnitude of these effects.

The conditional agreement between our *in vivo* measurement and *in vitro* models may lead to a question of whether an impairment of AR activity at its catalysis center (e.g., in ATase^{D173N,D175N}) would change the regulation of AT activity. AT and AR functional domains reside in one protein, and their interaction as well as regulation by effectors is rather complex. Therefore, we cannot totally rule out the possibility that *in vivo* regulation of AT activity in the *glnE475* mutant is different from that of the wild type. However, we disfavor such a complication for two reasons. First, it has been shown that the purified ATase$^{D173N,\ D175N}$ specifically abolishes AR activity without abrogating AT regulation by Gln and P_{II}.[27] Second, the two different AR$^-$ mutants, *glnE466* and *glnE475*, so far displayed identical phenotypes in all tests, including the $\tilde{n}$ values in a variety of nitrogen sources examined (Figs. 1–5, Table 1, and Table S4). This implies that either AT regulation is altered in an identical manner in the two genetically distinct ATase mutants or not at all. The theoretical refinement of the *in vitro* model we achieved will provide directions for experimental approaches in order to enhance quantitative understanding of ATase regulation and beyond. The central nitrogen metabolism in enteric bacteria is a compact system. It has many general features such as cyclic and allosteric regulations on gene expression and protein modification. With the depth of knowledge accumulated both *in vitro* and *in vivo* over ~40 years, it provides one of the most suited subsystems for demonstrative integration systems biology. A good example is the in-depth metabolomics and modeling study of ammonium assimilation in *E. coli* published recently.[22]

Costs associated with the benefit of counterbalancing a dynamic regulation

A cost issue may arise as the dynamic counterbalance is inevitably accompanied by a futile cycling. One adenylylation/deadenylylation cycle converts one ATP to AMP. The rate of ATP turnover was estimated for the wild-type ATase during cell growth in NH_4^+ as follows. Based on reported *in vitro* data, AT activity of the wild-type ATase is assumed to be no more than four times higher than that of the mutant ATase^{D173N,D175N} encoded by *glnE475*.[27] The measured internal concentrations of Gln, 2OG, and total GS subunits, and the $\tilde{n}$ value of the *glnE*$^+$ strain (FG1301) grown in NH_4^+, are 4.4 mM, 0.51 mM, 18 μM, and 5.9 (Fig. 3b and c and Table 1), respectively. Based on these, AT activities were calculated from our *in vitro* models. The resulting k_t^* ranged from 41 to 85 h^{-1}, which led to the

‡ The original interpretation of the published data is that the ADP-ribosylated portion could be a technical artifact formed during sampling.

corresponding rate of ATP turnover by the wild-type ATase being 6.0 to 12.5 μM/min. By substituting the growth rate of 0.66 h^{-1} (Fig. 4a) into the reported relationship for an *E. coli* W strain grown in continuous culture with glycerol as the sole carbon source,[47] the total ATP flux was estimated to be ~0.5 M/min. Therefore, ATP consumed in the futile cycling of the $glnE^+$ strain grown in NH_4^+ accounts for less than 0.003% of the total ATP generated. This *in vivo* originated calculation agrees with a previous estimate based on *in vitro* data.[16] Hence, the cost of maintaining the dynamic balance of GS activity is negligible compared to the cost for Gln synthesis. The latter consumes up to ~15% of the total ATP generated.[39]

A different cost issue arises in the context of system design. Is AR activity absolutely needed? Or could the cell "get by" with only AT activity (required during adaptation upon a nitrogen upshift) and compensation for the lack of AR activity by appropriately modifying the control of GS synthesis? At first sight, this strategy appears possible and attractive. The loss of AR activity, mainly affecting steady-state properties, can in principle be dealt with just by adjusting *glnA* transcriptional control. Such an alternative would reduce the evolutionary cost of maintaining the regulatory apparatus. However, this strategy would result in at least two problematic consequences. The first would be the additional cost of GS synthesis and possibly a selective mechanism for degradation of inactivated GS. Under normal regulations, GS amount is already a sizable fraction of cellular materials (1–2% of total protein). The second would be a requirement for an extremely low basal AT activity. As AT activity can only be balanced by GS synthesis in the absence of AR activity, it takes only a small fraction of the maximum AT activity to inactivate nearly all GS subunits in a cell doubling time. Having an extremely low basal AT activity in the steady state and still being able to be steeply activated at a time of need may be very difficult constraints to implement biochemically for an enzyme. *In vitro*, extremely low Gln plus high 2OG concentrations could be applied in the reconstituted system to achieve a low AT activity. *In vivo*, however, Gln and 2OG are metabolites that have to satisfy the needs of metabolism besides being allosteric effectors of ATase. Their dynamic ranges of concentration are limited. It may not be possible for them to regulate AT activity to a very low level *in vivo*. Because of the above, acquisition of the opposing AR activity may then be an expedient evolutionary strategy in order to deal with system control problems that would otherwise have led to irreversible GS adenylylation. We therefore expect dynamic counterbalance to be a generic control strategy for protein modification pathways.

Materials and Methods

Bacterial strains and cell growth

Isogenic *E. coli* K-12 strains used in this study are listed in Table S1. Cells were grown aerobically at 37 °C. For details of batch culture setup, media, and allele constructions, see SI Materials and Methods.

Metabolite pool measurements and β-galactosidase assay

Glu and Gln pools were extracted according to a "no-harvest" protocol and quantified after florescence derivatization with phthaldialdehyde followed by HPLC separation as described.[20,24] For 2OG pool, 1 ml of cells was collected through a fast filtration on a nylon membrane (25 -mm disc with 0.45 -μm pore size), immediately washed with 2 ml of medium, and extracted in 1 ml of 0.8 M $HClO_4$. This simple sampling protocol is adequate for batch cultures employed in this study where neither the nitrogen nor the carbon source is being significantly depleted. In other culturing situations, such as a sudden nutrient shift or in a nutrient-limited continuous culture, an alternative sampling protocol is required. Details of the different sampling protocols and their rationales will be presented elsewhere. Quantification of 2OG was achieved following a protocol of florescence derivatization with 4,5-methylenedioxy-1,2-phenylenediamine and HPLC separation.[48] Pool value in nanomoles per milliliter cells at 1 OD_{600} was converted directly into millimolar internal concentration. The conversion is based on 0.50 mg dry weight per milliliter cells per OD_{600} of the strains used in this study and ~2 μl of cellular water per milligram dry weight.[49,50] Samples of the *glnA-lacZ* strains were taken from cultures during exponential growth and at OD_{600} of around 0.2, 0.3, 0.4, and 0.5. β-Galactosidase assay was carried out as described and results are expressed as a differential rate of the *glnA* expression.[20,51]

GS assay

Permeabilized cells were prepared essentially as described.[52] Briefly, when OD_{600} of a culture in exponential growth reached ~0.4, the cells were mixed with 0.1 volume of 1 mg/ml hexadecyltrimethylammonium bromide (CTAB) at 37 °C for 2–3 min and incubated on ice for 20 min. The cells were then harvested, washed once with 1% KCl, and suspended in 1% KCl. γ-Glutamyl transferase activity was measured by the mixed imidazole assay system as described,[53,54] except that 0.1 mg/ml CTAB was included in the reaction mixture. Both activities in the presence of Mg^{2+}/Mn^{2+} and Mn^{2+} were determined, and $\tilde{n}$ value was calculated accordingly. The total concentration of GS subunit was calculated from a specific activity of purified enzyme in the presence of Mn^{2+}. There have been two reported specific activities of 100 and 56 μmol hydroxamic acid per minute per milligram GS.[53,54] The smaller value was applied; using the larger value has little effect on k_t values.

Acknowledgements

We thank Farwa Abid (Indiana University School of Medicine) for assistance during the early phase of genetic characterization. This research is supported by a research grant (RGP/P0022) from the Human Frontier Science Program. T.H. acknowledges support by the National Science Foundation (NSF) through grant MCB0746581. P.L. is grateful for the hospitality of the NSF Center for Theoretical Biological Physics at University of California at San Diego (grant PHY-0822283) where the quantitative research was initiated.

Supplementary Data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jmb.2010.09.046

References

1. Stadtman, E. R. (2004). Regulation of glutamine synthetase activity. In *EcoSal—Escherichia coli and Salmonella: Cellular and Molecular Biology* (Bock, A., Curtiss, R., III, Kaper, J. B., Neidhardt, F. C., Nystrom, T., Rudd, K. E. & Squires, C. L., eds), ASM Press, Washington, DC; http://www.ecosal.org.
2. Wulff, K., Mecke, D. & Holzer, H. (1967). Mechanism of the enzymatic inactivation of glutamine synthetase from *E. coli*. *Biochem. Biophys. Res. Commun.* **28**, 740–745.
3. Shapiro, B. M., Kingdon, H. S. & Stadtman, E. R. (1967). Regulation of glutamine synthetase. VII. Adenylyl glutamine synthetase: a new form of the enzyme with altered regulatory and kinetic properties. *Proc. Natl Acad. Sci. USA*, **58**, 642–649.
4. Kingdon, H. S., Shapiro, B. M. & Stadtman, E. R. (1967). Regulation of glutamine synthetase. 8. ATP: glutamine synthetase adenylyltransferase, an enzyme that catalyzes alterations in the regulatory properties of glutamine synthetase. *Proc. Natl Acad. Sci. USA*, **58**, 1703–1710.
5. Shapiro, B. M. (1969). The glutamine synthetase deadenylylating enzyme system from *Escherichia coli*. Resolution into two components, specific nucleotide stimulation, and cofactor requirements. *Biochemistry*, **8**, 659–670.
6. Anderson, W. B., Hennig, S. B., Ginsburg, A. & Stadtman, E. R. (1970). Association of ATP: glutamine synthetase adenylyltransferase activity with the P_1 component of the glutamine synthetase deadenylylation system. *Proc. Natl Acad. Sci. USA*, **67**, 1417–1424.
7. Jaggi, R., van Heeswijk, W. C., Westerhoff, H. V., Ollis, D. L. & Vasudevan, S. G. (1997). The two opposing activities of adenylyl transferase reside in distinct homologous domains, with intramolecular signal transduction. *EMBO J.* **16**, 5562–5571.
8. Jiang, P. & Ninfa, A. J. (2009). Reconstitution of *Escherichia coli* glutamine synthetase adenylyltransferase from N-terminal and C-terminal fragments of the enzyme. *Biochemistry*, **48**, 415–423.
9. van Heeswijk, W. C., Hoving, S., Molenaar, D., Stegeman, B., Kahn, D. & Westerhoff, H. V. (1996). An alternative P_{II} protein in the regulation of glutamine synthetase in *Escherichia coli*. *Mol. Microbiol.* **21**, 133–146.
10. Kamberov, E. S., Atkinson, M. R. & Ninfa, A. J. (1995). The *Escherichia coli* P_{II} signal transduction protein is activated upon binding 2-ketoglutarate and ATP. *J. Biol. Chem.* **270**, 17797–17807.
11. Jiang, P., Peliska, J. A. & Ninfa, A. J. (1998). The regulation of *Escherichia coli* glutamine synthetase revisited: role of 2-ketoglutarate in the regulation of glutamine synthetase adenylylation state. *Biochemistry*, **37**, 12802–12810.
12. Engleman, E. G. & Francis, S. H. (1978). Cascade control of *E. coli* glutamine synthetase. II. Metabolite regulation of the enzymes in the cascade. *Arch. Biochem. Biophys.* **191**, 602–612.
13. Brown, M. S., Segal, A. & Stadtman, E. R. (1971). Modulation of glutamine synthetase adenylylation and deadenylylation is mediated by metabolic transformation of the P_{II}-regulatory protein. *Proc. Natl Acad. Sci. USA*, **68**, 2949–2953.
14. Atkinson, M. R. & Ninfa, A. J. (1999). Characterization of the GlnK protein of *Escherichia coli*. *Mol. Microbiol.* **32**, 301–313.
15. van Heeswijk, W. C., Molenaar, D., Hoving, S. & Westerhoff, H. V. (2009). The pivotal regulator GlnB of *Escherichia coli* is engaged in subtle and context-dependent control. *FEBS J.* **276**, 3324–3340.
16. Segal, A., Brown, M. S. & Stadtman, E. R. (1974). Metabolite regulation of the state of adenylylation of glutamine synthetase. *Arch. Biochem. Biophys.* **161**, 319–327.
17. Rhee, S. G., Park, R., Chock, P. B. & Stadtman, E. R. (1978). Allosteric regulation of monocyclic interconvertible enzyme cascade systems: use of *Escherichia coli* glutamine synthetase as an experimental model. *Proc. Natl Acad. Sci. USA*, **75**, 3138–3142.
18. Jiang, P., Mayo, A. E. & Ninfa, A. J. (2007). *Escherichia coli* glutamine synthetase adenylyltransferase (ATase, EC 2.7.7.49): kinetic characterization of regulation by P_{II}, P_{II}-UMP, glutamine, and α-ketoglutarate. *Biochemistry*, **46**, 4133–4146.
19. Mura, U., Chock, P. B. & Stadtman, E. R. (1981). Allosteric regulation of the state of adenylylation of glutamine synthetase in permeabilized cell preparations of *Escherichia coli*. Studies of monocyclic and bicyclic interconvertible enzyme cascades, *in situ*. *J. Biol. Chem.* **256**, 13022–13029.
20. Ikeda, T. P., Shauger, A. E. & Kustu, S. (1996). *Salmonella typhimurium* apparently perceives external nitrogen limitation as internal glutamine limitation. *J. Mol. Biol.* **259**, 589–607.
21. Forchhammer, K., Hedler, A., Strobel, H. & Weiss, V. (1999). Heterotrimerization of P_{II}-like signalling proteins: implications for P_{II}-mediated signal transduction systems. *Mol. Microbiol.* **33**, 338–349.
22. Yuan, J., Doucette, C. D., Fowler, W. U., Feng, X. J., Piazza, M., Rabitz, H. A. *et al.* (2009). Metabolomics-

driven quantitative analysis of ammonia assimilation in *E. coli*. *Mol. Syst. Biol.* **5**, 302.
23. Yan, D., Ikeda, T. P., Shauger, A. E. & Kustu, S. (1996). Glutamate is required to maintain the steady-state potassium pool in *Salmonella typhimurium*. *Proc. Natl Acad. Sci. USA*, **93**, 6527–6531.
24. Kustu, S., Hirschman, J., Burton, D., Jelesko, J. & Meeks, J. C. (1984). Covalent modification of bacterial glutamine synthetase: physiological significance. *Mol. Gen. Genet.* **197**, 309–317.
25. Yan, D. (2007). Protection of the glutamate pool concentration in enteric bacteria. *Proc. Natl Acad. Sci. USA*, **104**, 9475–9480.
26. Xu, Y., Zhang, R., Joachimiak, A., Carr, P. D., Huber, T., Vasudevan, S. G. & Ollis, D. L. (2004). Structure of the N-terminal domain of *Escherichia coli* glutamine synthetase adenylyltransferase. *Structure*, **12**, 861–869.
27. Jiang, P., Pioszak, A. A. & Ninfa, A. J. (2007). Structure–function analysis of glutamine synthetase adenylyltransferase (ATase, EC 2.7.7.49) of *Escherichia coli*. *Biochemistry*, **46**, 4117–4132.
28. Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M. *et al.* (2006). Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. *Mol. Syst. Biol.* **2**, 2006.0008.
29. Kang, Y., Durfee, T., Glasner, J. D., Qiu, Y., Frisch, D., Winterberg, K. M. & Blattner, F. R. (2004). Systematic mutagenesis of the *Escherichia coli* genome. *J. Bacteriol.* **186**, 4921–4930.
30. Muse, W. B. & Bender, R. A. (1992). Map position of the *glnE* gene from *Escherichia coli*. *J. Bacteriol.* **174**, 7876–7877.
31. Schmitz, R. A. (2000). Internal glutamine and glutamate pools in *Klebsiella pneumoniae* grown under different conditions of nitrogen availability. *Curr. Microbiol.* **41**, 357–362.
32. van Heeswijk, W. C., Rabenberg, M., Westerhoff, H. V. & Kahn, D. (1993). The genes of the glutamine synthetase adenylylation cascade are not regulated by nitrogen in *Escherichia coli*. *Mol. Microbiol.* **9**, 443–457.
33. Csonka, L. N., Ikeda, T. P., Fletcher, S. A. & Kustu, S. (1994). The accumulation of glutamate is necessary for optimal growth of *Salmonella typhimurium* in media of high osmolality but not induction of the *proU* operon. *J. Bacteriol.* **176**, 6324–6333.
34. Atkinson, M. R. & 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.
35. Bancroft, S., Rhee, S. G., Neumann, C. & Kustu, S. (1978). Mutations that alter the covalent modification of glutamine synthetase in *Salmonella typhimurium*. *J. Bacteriol.* **134**, 1046–1055.
36. Chock, P. B. & Stadtman, E. R. (1977). Superiority of interconvertible enzyme cascades in metabolite regulation: analysis of multicyclic systems. *Proc. Natl Acad. Sci. USA*, **74**, 2766–2770.
37. Stadtman, E. R. & Chock, P. B. (1977). Superiority of interconvertible enzyme cascades in metabolic regulation: analysis of monocyclic systems. *Proc. Natl Acad. Sci. USA*, **74**, 2761–2765.
38. Shacter, E., Chock, P. B. & Stadtman, E. R. (1984). Regulation through phosphorylation/dephosphorylation cascade systems. *J. Biol. Chem.* **259**, 12252–12259.
39. Reitzer, L. (2003). Nitrogen assimilation and global regulation in *Escherichia coli*. *Annu. Rev. Microbiol.* **57**, 155–176.
40. Ninfa, A. J. & Magasanik, B. (1986). Covalent modification of the *glnG* product, NRI, by the *glnL* product, NRII, regulates the transcription of the *glnALG* operon in *Escherichia coli*. *Proc. Natl Acad. Sci. USA*, **83**, 5909–5913.
41. Keener, J. & 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.
42. Feng, J., Atkinson, M. R., McCleary, W., Stock, J. B., Wanner, B. L. & Ninfa, A. J. (1992). Role of phosphorylated metabolic intermediates in the regulation of glutamine synthetase synthesis in *Escherichia coli*. *J. Bacteriol.* **174**, 6061–6070.
43. Blauwkamp, T. A. & Ninfa, A. J. (2002). Nac-mediated repression of the *serA* promoter of *Escherichia coli*. *Mol. Microbiol.* **45**, 351–363.
44. Nordlund, S. & Ludden, P. W. (2004). Post-translational regulation of nitrogenase in photosynthetic bacteria. In *Genetics and Regulation of Nitrogen Fixation in Free-Living Bacteria* (Klipp, W., Masepohl, B., Gallon, J. R. & Newton, W. E., eds), pp. 175–196, Kluwer Academic Publishers, Dordrecht.
45. Zhang, Y., Pohlmann, E. L., Ludden, P. W. & Roberts, G. P. (2001). Functional characterization of three GlnB homologs in the photosynthetic bacterium *Rhodospirillum rubrum*: roles in sensing ammonium and energy status. *J. Bacteriol.* **183**, 6159–6168.
46. Liang, J. H., Nielsen, G. M., Lies, D. P., Burris, R. H., Roberts, G. P. & Ludden, P. W. (1991). Mutations in the *draT* and *draG* genes of *Rhodospirillum rubrum* result in loss of regulation of nitrogenase by reversible ADP-ribosylation. *J. Bacteriol.* **173**, 6903–6909.
47. Farmer, I. S. & Jones, C. W. (1976). The energetics of *Escherichia coli* during aerobic growth in continuous culture. *Eur. J. Biochem.* **67**, 115–122.
48. Wang, Z. J., Zaitsu, K. & Ohkura, Y. (1988). High-performance liquid chromatographic determination of α-keto acids in human serum and urine using 1,2-diamino-4,5-methylenedioxybenzene as a precolumn fluorescence derivatization reagent. *J. Chromatogr.* **430**, 223–231.
49. Cayley, S., Lewis, B. A., Guttman, H. J. & Record, M. T., Jr. (1991). Characterization of the cytoplasm of *Escherichia coli* K-12 as a function of external osmolarity. Implications for protein–DNA interactions in vivo. *J. Mol. Biol.* **222**, 281–300.
50. Stock, J. B., Rauch, B. & Roseman, S. (1977). Periplasmic space in *Salmonella typhimurium* and *Escherichia coli*. *J. Biol. Chem.* **252**, 7850–7861.
51. Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
52. Bender, R. A., Janssen, K. A., Resnick, A. D., Blumenberg, M., Foor, F. & Magasanik, B. (1977). Biochemical parameters of glutamine synthetase from *Klebsiella aerogenes*. *J. Bacteriol.* **129**, 1001–1009.

53. Stadtman, E. R., Ginsburg, A., Ciardi, J. E., Yeh, J., Hennig, S. B. & Shapiro, B. M. (1970). Multiple molecular forms of glutamine synthetase produced by enzyme catalyzed adenylation and deadenylylation reactions. *Adv. Enzyme. Regul.* **8**, 99–118.

54. Stadtman, E. R., Smyrniotis, P. Z., Davis, J. N. & Wittenberger, M. E. (1979). Enzymic procedures for determining the average state of adenylylation of *Escherichia coli* glutamine synthetase. *Anal. Biochem.* **95**, 275–285.