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.

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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,
encoded by \textit{glnA}), glutamate synthase (GOGAT, encoded by \textit{gltBD}), and glutamate dehydrogenase (GDH, encoded by \textit{gdhA}).

\[
\begin{align*}
\text{Glu} + \text{NH}_3 + \text{ATP} & \xrightarrow{\text{GS}} \text{Gln} + \text{ADP} + \text{P}_i \\
2\text{OG} + \text{Gln} + \text{NADPH} + \text{H}^+ & \xrightarrow{\text{GOGAT}} 2\text{Glu} + \text{NADP}^+ \\
2\text{OG} + \text{NH}_3 + \text{NADPH} + \text{H}^+ & \xrightarrow{\text{GDH}} \text{Glu} + \text{NADP}^+
\end{align*}
\]

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 \textit{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 \textit{Mg}\(^{2+}\).\(^2\,\,^2\,\,^4\) Both of the adenylylation and deadenylylation reactions require catalysis by a single but bifunctional adenylyltransferase/adenylyl-removing enzyme (ATase, encoded by \textit{glnE})\(^2\,\,^4\,\,^6\).

\[
\text{GS} + \text{ATP} \xrightarrow{\text{ATase (adenylyltransferase activity)}} \text{GS-AMP} + \text{PP}_i \quad \text{and} \\
\text{GS-AMP} + \text{P}_i \xrightarrow{\text{ATase (adenylyl-removing activity)}} \text{GS} + \text{ADP}.
\]

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 \textit{P}_\text{II} and its paralogue GlnK (Fig. S1).\(^4\,\,^5\,\,^9\,\,^2\,\,^1\,\,^5\,\,^1\,\,^6\) In vitro, Gln acts directly on ATase and indirectly through \textit{P}_\text{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 \textit{P}_\text{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 and in permeabilized cells.\(^1\,\,^1\,\,^6\,\,^1\,\,^8\) 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.\(^1\,\,^2\) However, the regulatory mechanisms established from in vitro studies do not necessarily all operate in vivo.\(^2\,\,^1\,\,^1\) 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.\(^2\) 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.\(^2\,\,^3\) 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 Gln 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 \textit{Escherichia coli}. We demonstrate that an imbalance of the AT and AR activities in \textit{AR}/\textit{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.\(^1\) The quantitatively derived results are obtained by developing another mathematical model to capture the in vivo 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 \textit{P}_\text{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 \(AgltD\) mutant on minimal media containing arginine (Fig. 1; comparing the \(AgltD glnE475\) 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, \(glnE::Tn5-KAN-I-SceI\), from the Blattner knockout collection;\(^{29}\) and another early reported insertion mutation, \(glnE::Tn5-131\).\(^{30}\) 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

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**Fig. 1.** AR\(^{-}\) mutations suppress the growth defect of a \(AgltD\) mutant with arginine as the sole nitrogen source. Colonies of FG1294 (\(AgltD ΔyqiK::kan\)), FG1312 (\(AgltD glnE466 ΔyqiK::kan\)), and FG1438 (\(AgltD glnE475 ΔyqiK::kan\)) were grown on agar medium for 4 days. The scale bar represents 0.5 mm.
source. Upon an NH₄⁺ 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 NH₄⁺ upshift. Growth delay was also observed when other poor nitrogen sources such as arginine or threonine were used in the preshift cultures. The glnE::Tn5-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 NH₄⁺ 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 NH₄⁺ medium, free of NH₄⁺ 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 NH₄⁺ 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 NH₄⁺ 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 NH₄⁺ medium (Fig. 3c; black bars). As expected, no GS adenylylation was observed in the AghnE strain (FG1320) where the glnE coding region is completely eliminated. 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 AghnE (FG1320), glnE::Tn5-KAN-I-SceI (FG1413), and glnE::Tn5-151 (FG1411), the Gln pools were similar to those in the glnE⁺ strains at OD₆₀₀ ~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.
subunits per GS dodecamer complex, \( n \), being 5.9 and 4.5. In the AR\(^-\) mutants (FG1468 and FG1469), however, GS was excessively adenylylated, with \( n \) 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...
was expected, as it has been reported that ATase is dispensable during steady-state growth regardless of external nitrogen availability. 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 \( \text{NH}_4^+ \) medium. However, we found that the Gln pools were almost

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**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 (A\( glnE \)), compared to that of FG1301 in (a).

**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 ARmutants 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 NH3 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 values of the ARmutants from proline culture are smaller than those from NH3 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 ARmutants 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

\[ \frac{dx_a}{dt} = \frac{\kappa_t e_t x_u}{(K_u + x_u)} - \kappa_t e_t x_a / (K_a + x_a) - \lambda x_a \]  
\[ \frac{dx_u}{dt} = \kappa_t e_t x_a / (K_a + x_a) - \kappa_t e_t x_u / (K_u + x_u) - \lambda x_u + \gamma \]  

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 \( \kappa_a \), and deadenylylation at rate \( \kappa_r \). The latter is taken to be zero for the ARmutant.

The last term in Eq. (1) describes the dilution due to cellular growth at growth rate \( \lambda \). \( \gamma \) denotes the rate of GS synthesis, which, in the absence of significant degradation, is just \( \lambda x_t \), \( x_t = x_a + x_u \) is the concentration of all GS subunits.

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

\[ k_t = \frac{\bar{n} \lambda}{12 - \bar{n}} + \frac{\bar{n} \lambda x_1}{12 K_u} \]  

where the adenylylation number \( \bar{n} = 12 x_a / (x_u + x_a) \), the total GS amount \( x_u \), and the growth rate \( \lambda \) are all measurable quantities. The in vivo value of \( K_{wu} \) is uncertain. In the following, we apply one reported in vitro value, \( K_{wu} = 35 \) \( \mu \)M.18 Another available but much larger \( K_{wu} \) 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 \( \bar{n} \), \( x_a \), and \( \lambda \) for the ARmutants 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 \( \bar{n} \) only varied between ~7 and ~11.) The results indicate that AT activity is significantly regulated in the ARmutants 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 PII (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 PII and Gln has recently been characterized in vitro by Jiang et al.16 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
study on the dependence of PII urydylation 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–PII–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 PII-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 PII levels.[11]

In the in vitro models, the adenylylation flux is given by \( \kappa e^* x_u / (K_u + x_u) \), where \( e^* \) is the in vitro ATase concentration, and the in vitro rate \( \kappa^* \) 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 \( \kappa^* \) deduced above, \( \kappa^* = \kappa^* e^* / K_u \), 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, \( e^* \) is the only parameter adjusted to minimize the differences between \( \kappa^* \) and \( k_t \) (weighted by the uncertainty in \( k_t \)). Values of the best-fit parameter \( e^* \) 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

![Fig. 6. AT activity as a function of metabolite pool sizes.](image)

<table>
<thead>
<tr>
<th>Nitrogen source(^a)</th>
<th>Growth rate (h(^{-1}))</th>
<th>( \hat{n} )</th>
<th>Total GS subunit (( \mu )M)</th>
<th>( k_t ) (h(^{-1}))</th>
<th>Gln pool (mM)</th>
<th>2OG pool (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium</td>
<td>0.60±0.01</td>
<td>11.1±0.0</td>
<td>103±3</td>
<td>8.9±1.3</td>
<td>2.45±0.14</td>
<td>0.94±0.07</td>
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<tr>
<td>Cytidine</td>
<td>0.51±0.01</td>
<td>11.0±0.0</td>
<td>90±3</td>
<td>6.7±0.5</td>
<td>2.09±0.02</td>
<td>3.18±0.06</td>
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<tr>
<td>Alanine</td>
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<td>10.3±0.0</td>
<td>89±5</td>
<td>4.1±0.4</td>
<td>0.95±0.01</td>
<td>6.00±0.43</td>
</tr>
<tr>
<td>Gly+Arg</td>
<td>0.50±0.00</td>
<td>10.4±0.0</td>
<td>92±4</td>
<td>4.5±0.3</td>
<td>1.73±0.09</td>
<td>2.81±0.14</td>
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<tr>
<td>Aspartate</td>
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<td>7.6±0.1</td>
<td>104±4</td>
<td>1.7±0.2</td>
<td>0.40±0.15</td>
<td>1.28±0.03</td>
</tr>
<tr>
<td>Proline</td>
<td>0.39±0.00</td>
<td>7.3±0.1</td>
<td>129±1</td>
<td>1.5±0.1</td>
<td>0.22±0.03</td>
<td>1.58±0.26</td>
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<td>Glycine</td>
<td>0.24±0.00</td>
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<td>Arginine</td>
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<tr>
<td>Glutamate</td>
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<td>119±1</td>
<td>0.5±0.0</td>
<td>0.05±0.01</td>
<td>1.55±0.09</td>
</tr>
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</table>

\( a \) 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.
results in a total loss of AT activity. The insertion mutations glnE::Tn5-KAN-I-SceI and glnE::Tn5-151 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 NH₄⁺ upshift and downshift. Because protein modification is a faster response compared to gene expression, it is logical that ATase is constitutively expressed 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. 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 NH₄⁺ 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 deamination, 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). Serves as our current working hypothesis, one possible explanation is that the poor nitrogen sources could only supply limited NH₄⁺ 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 NH₄⁺ concentration is difficult. As 20 μM external NH₄⁺ may still support a growth almost as fast as 10 mM NH₄⁺, internal NH₄⁺ 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 n values in Table 1 and Table S4) may still leave enough active enzymes to saturate the trace amount of internal NH₄⁺ in concentrations and synthesize Gln at the same rate as in the wild type.

**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, 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 results in a total loss of AT activity. The insertion mutations glnE::Tn5-KAN-I-SceI and glnE::Tn5-151 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 NH₄⁺ upshift and downshift. Because protein modification is a faster response compared to gene expression, it is logical that ATase is constitutively expressed 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. 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.

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$glnE466$ was isolated as a mutation that suppresses the growth defect of a GOGAT$^{-}$ mutant grown with trace amount of NH$_4^+$. 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. Another characteristic of the GOGAT$^{-}$ mutants is their abnormally high Gln pool. Previously, we identified two types of suppressor mutation arisen from GOGAT$^{-}$ mutants. 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 Gln pool in a GOGAT$^{-}$ background. The $glnA$ mutation also shows a higher than normal Glu 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 vitro observations. 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. Although such capacities were examined both in vitro and in situ, 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 $\sim$20-fold decrease in AT activity results in only $\sim$30% drop in the degree of adenylylation (in; Table 1). Alternatively, a 10-fold drop in $n$ 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. Its phosphorylation is achieved by the kinase function of the bifunctional NtrB, or through autophosphorylation by small phosphor donors such as acetyl phosphate. 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. 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 dinitrogen reductase by ADP ribosylation in response to the availability of fixed nitrogen and/or light level. 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 PII analogous to ATase. 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 N₂-fixing condition, but none was modified in the wild type. 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 (unadenylated) GS during growth. The result reveals regulation of AT activity in *E. coli* 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 PII 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 PII stimulation. 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*) 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* specifically abolishes AR activity without abrogating AT regulation by Gln and PII. Second, the two different AR mutants, *glnE466* and *glnE475*, so far displayed identical phenotypes in all tests, including the *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.

**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 NH₄⁺ 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* encoded by *glnE475*. The measured internal concentrations of Gln, 2OG, and total GS subunits, and the *n* value of the *glnE¹* strain (FG1301) grown in NH₄⁺, 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 *kₚ* ranged from 41 to 85 h⁻¹, which led to the

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†The original interpretation of the published data is that the ADP-ribosylated portion could be a technical artifact formed during sampling.
Dynamic Regulation of GS Modification in E. coli

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. For 2OG pool, 1 ml of cells was collected through a fast filtration on a nylon membrane (25 -μm disc with 0.45 -μm pore size), immediately washed with 2 ml of medium, and extracted in 1 ml of 0.8 M HClO4. 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. Pool value in nanomoles per milliliter cells at 1 OD600 was converted directly into millimolar internal concentration. The conversion is based on 0.50 mg dry weight per milliliter conversion is based on 0.50 mg dry weight per milliliter dry weight. Samples of the glnA-lacZ strains were taken from cultures during exponential growth and at OD600 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.

GS assay

Permeabilized cells were prepared essentially as described. Briefly, when OD600 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, except that 0.1 mg/ml CTAB was included in the reaction mixture. Both activities in the presence of Mg2+/Mn2+ and Mn2+ were determined, and n value was calculated accordingly. The total concentration of GS subunit was calculated from a specific activity of purified enzyme in the presence of Mn2+. There have been two reported specific activities of 100 and 56 μmol hydroxamic acid per minute per milligram GS. The smaller value was applied; using the larger value has little effect on kcat values.

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, 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 NH4 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. 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.

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.

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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

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