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Reciprocal regulation of the Autophosphorylation of Enzyme I^{Ntr} by Glutamine and α-Ketoglutarate in *Escherichia coli*

Chang-Ro Lee[a,d], **Young-Ha Park**[a], **Miri Kim**[a], **Yeon-Ran Kim**[a], **Soyoung Park**[b], **Alan Peterkofsky**[c], and **Yeong-Jae Seok**[a,b,1]

[a]Department of Biological Sciences and Institute of Microbiology, Seoul National University, Republic of Seoul 151-742, Korea

[b]Department of Biophysics and Chemical Biology, Seoul National University, Seoul 151-742, Republic of Korea

[c]Laboratory of Cell Biology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892, USA

[d]Department of Biological Sciences, Myongji University, Yongin, Gyeonggido 449-728, Republic of Korea

Summary

In addition to the phosphoenolpyruvate:sugar phosphotransferase system (sugar PTS), most proteobacteria possess a paralogous system (nitrogen phosphotransferase system, PTS^{Ntr}). The first proteins in both pathways are enzymes (enzyme I^{sugar} and enzyme I^{Ntr}) that can be autophosphorylated by phosphoenolpyruvate. The most striking difference between enzyme I^{sugar} and enzyme I^{Ntr} is the presence of a GAF domain at the N-terminus of enzyme I^{Ntr}. Since the PTS^{Ntr} was identified in 1995, it has been implicated in a variety of cellular processes in many proteobacteria and many of these regulations have been shown to be dependent on the phosphorylation state of PTS^{Ntr} components. However, there has been little evidence that any component of this so-called PTS^{Ntr} is directly involved in nitrogen metabolism. Moreover, a signal regulating the phosphorylation state of the PTS^{Ntr} had not been uncovered. Here, we demonstrate that glutamine and α-ketoglutarate, the canonical signals of nitrogen availability, reciprocally regulate the phosphorylation state of the PTS^{Ntr} by direct effects on enzyme I^{Ntr} autophosphorylation and the GAF signal transduction domain is necessary for the regulation of enzyme I^{Ntr} activity by the two signal molecules. Taken together, our results suggest that the PTS^{Ntr} senses nitrogen availability.

Keywords

GAF domain; glutamine; α–ketoglutarate; nitrogen PTS; phosphorylation-dependent mobility shift

Introduction

How cells respond to environmental (extracellular) signals is of fundamental importance in biology. Most organisms, including bacteria, use a signal transduction pathway to deliver an extracellular signal to some intracellular target. Many of the relay molecules in signal

[1]To whom correspondence should be addressed: Tel: 82-2-880-8827; Fax: 82-2-888-4911; yjseok@snu.ac.kr.

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transduction pathways are protein kinases that create a “phosphorylation cascade”. The sugar phosphotransferase system (PTS) in bacteria is a group translocation system whose relay proceeds sequentially from PEP to Enzyme I, HPr, EIIA, EIIB, and finally to the incoming sugar that is transported through EIIC in the membrane and concomitantly phosphorylated (Deutscher *et al.*, 2006). In addition to its primary functions in sugar uptake and phosphorylation, this complex protein system carries out numerous regulatory functions such as chemoreception (Lux *et al.*, 1995), activation of glycogen phosphorylase by HPr (Seok *et al.*, 1997), inhibition of non-PTS sugar permeases (Deutscher *et al.*, 2006), activation of pyruvate decarboxylase (FrsA) by $EIIA^{Glc}$ (Koo *et al.*, 2004; Lee *et al.*, 2011), activation of adenylyl cyclase by phospho-$EIIA^{Glc}$ (Park *et al.*, 2006) and regulation of Mlc activity to control the expression level of the carbohydrate PTS and related proteins (Lee *et al.*, 2000; Nam *et al.*, 2001; Tanaka *et al.*, 2000).

Analysis of the *Escherichia coli* genome has revealed a parallel PTS that has been referred to as the nitrogen PTS (PTS^{Ntr}); it consists of EI^{Ntr} encoded by *ptsP*, NPr encoded by *ptsO*, and $EIIA^{Ntr}$ encoded by *ptsN* which are paralogues of the carbohydrate PTS components EI, HPr, and EIIA, respectively (Peterkofsky *et al.*, 2006; Pflüger-Grau and Görke, 2010; Powell *et al.*, 1995). Since phosphoryl transfer to a specific substrate has not yet been demonstrated for the PTS^{Ntr}, it has been suggested that this system functions mainly in a regulatory capacity (Reizer *et al.*, 1996). The PTS^{Ntr} has been implicated in poly-β-hydroxybutyrate accumulation and nitrogen fixation in *Azotobacter vinelandii* (Segura and Espin, 1998), virulence in *Legionella pneumophila* (Higa and Edelstein, 2001) and *Salmonella enterica* (Choi *et al.*, 2010), melanin synthesis and nitrogen fixation in *Rhizobium etli* (Michiels *et al.*, 1998), regulation of ATP-dependent transporters in *Rhizobium leguminosarum* (Prell *et al.*, 2012) and pulmonary infection (Zhang *et al.*, 2005) and carbon source-mediated inhibition of the σ^{54}-dependent *Pu* promoter of the TOL plasmid in *Pseudomonas* species (Cases *et al.*, 2001). In *E. coli*, $EIIA^{Ntr}$ was shown to be involved in the regulation of the essential GTPase, Era, which appears to function in cell cycle progression and the initiation of cell division (Powell *et al.*, 1995), although the mechanism of this regulation has not yet been defined.

Recently, a direct role of the *E. coli* PTS^{Ntr} in regulation of K^+ uptake was described. Dephosphorylated $EIIA^{Ntr}$ binds to and regulates the low-affinity K^+ transporter TrkA (Lee *et al.*, 2007) and the K^+-dependent sensor kinase KdpD (Lüttmann *et al.*, 2009; Pflüger-Grau and Görke, 2010). In the absence of dephosphorylated $EIIA^{Ntr}$, K^+ uptake through TrkA increases, resulting in unusually high intracellular K^+ concentrations. K^+ regulates global gene expression involving both σ^{70}- and σ^{S}-dependent promoters (Lee *et al.*, 2010). Furthermore, dephosphorylated $EIIA^{Ntr}$ was shown to modulate the phosphate starvation response through interaction with the sensor kinase PhoR (Lüttmann *et al.*, 2012). A physiological role of NPr was also shown. The dephosphorylated form of NPr interacts with and inhibits LpxD, which catalyzes biosynthesis of lipid A of the lipopolysaccharide (LPS) layer (Kim *et al.*, 2011).

Although several regulatory interactions involving proteins of the PTS^{Ntr} have been shown to be dependent on the phosphorylation state of PTS^{Ntr} components, the connection between them was obscure. The purpose of this study was to search for a signal regulating the phosphorylation state of the PTS^{Ntr}; this might serve as the central feature of the overall metabolic regulation by this system.

The mobility of many eukaryotic proteins is shifted on SDS-PAGE when they become phosphorylated (Zhou *et al.*, 2000); we refer to this as a phosphorylation-dependent mobility shift (PDMS). This PDMS is also observed with some proteins in bacteria (Lee and Helmann, 2006; Nam *et al.*, 2005). While $EIIA^{Glc}$ shows a significant PDMS on SDS-PAGE

(Hogema *et al.*, 1998), $EIIA^{Ntr}$ does not. By comparing amino acid sequences near the phosphorylation sites of $EIIA^{Glc}$ and $EIIA^{Ntr}$, site-directed mutagenesis of $EIIA^{Ntr}$ produced a species exhibiting a PDMS; the PDMS of this form was used to identify the signals (glutamine and α-ketoglutarate) regulating the phosphorylation state of the PTS^{Ntr}. The primary site of the regulation was identified to be the GAF domain of EI^{Ntr}.

Results

Engineering $EIIA^{Ntr}$ to exhibit a phosphorylation-dependent mobility shift (PDMS) on SDS-PAGE

$EIIA^{Glc}$ has previously been observed to change its mobility on SDS-PAGE upon phosphorylation of its active site histidine (Hogema *et al.*, 1998), whereas $EIIA^{Ntr}$ does not exhibit such a PDMS (Fig. 1A). This PDMS property has been an important asset in studying the regulatory functions of $EIIA^{Glc}$ that depend on its state of phosphorylation. The focus of the current study was to define the mechanism for the various regulatory functions of $EIIA^{Ntr}$. The first step in the study was to modify the structure of $EIIA^{Ntr}$ so that it exhibits a PDMS. A comparison of the amino acid sequences surrounding the phosphorylation site of $EIIA^{Glc}$ and $EIIA^{Ntr}$ revealed that, while $EIIA^{Glc}$ has two negatively charged amino acids (E86 and D94) situated on both sides of the phosphorylation site (H90), $EIIA^{Ntr}$ has three negatively charged amino acids clustered on one side of the phosphorylation site (H73) (Fig. S1A). To define the possible importance of the negatively charged amino acid E86 for the PDMS of $EIIA^{Glc}$, the E86A mutant was generated. Fig. S1B demonstrates the requirement of E86 for the PDMS of $EIIA^{Glc}$ in spite of the fact that $EIIA^{Glc}$(E86A) can still be phosphorylated (Fig. S1C). To further explore the importance of the distribution of the negatively charged amino acids around the phosphorylation site for the PDMS, we performed systematic site-directed mutagenesis in the region of residues 69 to 76 of $EIIA^{Ntr}$ by changing each residue, one at a time, to aspartate. Each mutant protein was expressed and tested for solubility and the PDMS on SDS-PAGE. Although all of the purified mutant proteins showed a measurable PDMS on SDS-PAGE (Fig. S2), only $EIIA^{Ntr}$(K75D) was as soluble (did not make an inclusion body) and as phosphorylatable as the wild-type protein (Figs. 1A and S2). Therefore, we selected $EIIA^{Ntr}$(K75D) as a good candidate to search for the signaling molecule(s) regulating the phosphorylation state of the PTS^{Ntr}.

To validate the use of this mutant form of $EIIA^{Ntr}$, we tested whether it behaves as does the wild type protein in an *in vivo* function test. It was previously demonstrated that deletion of *ptsN* (encoding $EIIA^{Ntr}$) resulted in Ala-Leu dipeptide-dependent growth inhibition of *E. coli* in M9 medium containing glucose as a carbon source and that the growth inhibition was neutralized by ectopic expression of $EIIA^{Ntr}$ (Lee *et al.*, 2005). Expression of $EIIA^{Ntr}$(K75D) in the *ptsN* mutant also restored resistance to the Ala-Leu dipeptide-dependent growth inhibition to a level similar to the wild type protein (Fig. 1B), indicating that $EIIA^{Ntr}$(K75D) can substitute for the wild type protein *in vivo*.

E. coli cells expressing $EIIA^{Ntr}$(K75D) were tested for the PDMS under conditions known to influence the *in vivo* phosphorylation state of $EIIA^{Ntr}$. Similar to previous observations (Bahr *et al.*, 2011; Pflüger and de Lorenzo, 2007), only the dephosphorylated form of $EIIA^{Ntr}$ could be detected in an *E. coli* mutant deleted for the *ptsP* gene encoding EI^{Ntr}, while $EIIA^{Ntr}$ exists mainly in a phosphorylated form in cells expressing the *ptsP* gene grown in LB medium (Fig. 2A). This provides further evidence for the adequacy of the K75D mutant of $EIIA^{Ntr}$ as a phosphorylation-state probe.

It has previously been shown that the phosphorylation state of $EIIA^{Ntr}$ in *Pseudomonas putida* could be altered by nitrogen sources. The phosphorylated form of $EIIA^{Ntr}$ was more

abundant than its dephosphorylated form in cells grown with nitrate as the sole nitrogen source, whereas the dephosphorylated form increased in cells grown in the presence of ammonium salts (Pflüger and de Lorenzo, 2007). Because *E. coli* cells cannot use nitrate as a nitrogen source, we employed alanine as a poor nitrogen source. The left panel of Fig. 2B shows that the dephosphorylated form of $EIIA^{Ntr}$ prevails in *E. coli* cells supplemented with 20 mM ammonium ion, whereas exposure of cells to the same concentration of the poorer nitrogen source favors the phosphorylated state, similar to the case in *P. putida*. These *in vivo* phosphorylation studies further validate the use of $EIIA^{Ntr}$(K75D) as a probe to explore the regulatory aspects of the PTS^{Ntr}.

Reciprocal regulation by glutamine and α–ketoglutarate of autophosphorylation of EI^{Ntr}; dependence on the GAF domain

Taking advantage of the unique PDMS exhibited by $EIIA^{Ntr}$(K75D), numerous factors were screened for a signal(s) affecting the phosphorylation state of the nitrogen PTS. We have previously shown that the PTS^{Ntr} regulates the sensitivity to serine, isoleucine, leucine and leucine-containing peptides of an *E. coli* K-12 strain harboring a frameshift mutation in the *ilvG* gene (Lee *et al.*, 2005) and that expression of several genes involved in amino acid metabolism was significantly influenced by the *ptsN* deletion (Lee *et al.*, 2010). Therefore, we explored the possibility that an amino acid might regulate the phosphorylation state of the PTS^{Ntr}. Interestingly, out of the 20 amino acids tested, only glutamine showed an apparent inhibitory effect on the phosphorylation state of $EIIA^{Ntr}$(K75D) (Fig. S3).

Previous studies have established that cellular glutamine and α-ketoglutarate levels sense nitrogen availability in opposite directions and that they regulate the activity of glutamine synthetase antagonistically through GlnD and GlnE of the *bona fide* Ntr signal transduction system (Ninfa and Atkinson, 2000). Therefore, we also tested the effect of α-ketoglutarate on the phosphotransferase activity of the PTS^{Ntr}. The data of Figs. 3 and 4 demonstrate that glutamine (Gln) and α–ketoglutarate (α-KG) oppositely affect the *in vitro* phosphorylation of $EIIA^{Ntr}$(K75D); glutamine inhibits and α–ketoglutarate stimulates. Both the inhibitory and stimulatory effects depend on the presence of the GAF domain of EI^{Ntr}. The data in Fig. 4 indicate that both the inhibitory effect of glutamine and the stimulatory effect of α–ketoglutarate are concentration-dependent. Noteworthy was the finding that, while a form of EI^{Ntr} deleted for the GAF domain supported phosphotransfer from PEP, there was no effect of either compound at any of the concentrations used. The requirement of the GAF domain for the activation or inhibition effects pointed to EI^{Ntr} as the locus of the effect. The data in Fig. 5 validate this prediction. Autophosphorylation by [^{32}P]PEP of EI^{Ntr} is inhibited by glutamine (Fig. 5A) and stimulated by α–ketoglutarate (Fig. 5B). EI^{Ntr} lacking the GAF domain was insensitive to the low molecular weight effectors.

The specificity for the interaction of glutamine and α–ketoglutarate with EI^{Ntr} was studied by quenching of tryptophan fluorescence (Fig. 6). While there was a concentration-dependent decrease in the fluorescence by either glutamine or α–ketoglutarate, the fluorescence spectrum was unaffected by glutamic acid. The calculated *K*d for glutamine was 0.7 mM and that for α–ketoglutarate was 3.3 mM. A similar study carried out with EI^{Ntr} lacking the GAF domain showed no effect on the fluorescence spectrum of any of the three low molecular weight compounds (Fig. S4). Physiological concentrations of α-ketoglutarate were reported to be between 0.14 and 0.91 mM in *E. coli* cells grown in the presence of excess ammonium salt (Senior, 1975) but were higher than 10 mM in the presence of poor nitrogen sources (Doucette *et al.*, 2011). While the concentration of glutamine is very low in cells under nitrogen-limiting conditions, it abruptly increases to higher than 10 mM with a concomitant decrease in the α-ketoglutarate pool to below 1 mM when cells were subjected to ammonium upshift (Doucette *et al.*, 2011). Therefore, the *K*d values for binding of α-ketoglutarate and glutamine to EI^{Ntr} determined in this study

suggest that the observed effects of these two metabolites on the phosphorylation state of the PTS^{Ntr} are physiologically relevant. Then we compared the effect of glutamine and α-ketoglutarate at 10 mM on the velocity of the phosphotransferase reaction (Fig. 7). When we measured the phosphorylation level of $EIIA^{Ntr}$ in the presence of trace amounts of EI^{Ntr} and NPr as a function of reaction time, about 1/3 of $EIIA^{Ntr}$ was phosphorylated in 1.5 min in the reaction without any effector (control) while it took 4 min to get the same level of $EIIA^{Ntr}$ phosphorylation in the reaction containing 10 mM glutamine. In contrast, in the presence of 10 mM α-ketoglutarate, $EIIA^{Ntr}$ was essentially completely phosphorylated in 2 min. Because intracellular concentrations of glutamine and α-ketoglutarate are reciprocally regulated in wild-type *E. coli* cells, the data in Fig. 7 imply that the reciprocal regulation of the autophosphorylation of EI^{Ntr} by α-ketoglutarate and glutamine can result in significant difference (more than 5 times) in the phosphotransferase reaction velocity of the PTS^{Ntr} in response to nitrogen availability *in vivo*. To obtain more accurate steady-state kinetics associated with the regulation of the phosphorylation state of the PTS^{Ntr}, the mechanism of PTS^{Ntr} dephosphorylation needs to be identified.

The GAF domain of EI^{Ntr} senses nitrogen availability *in vivo*

The canonical signal of nitrogen limitation in enteric bacteria is the intracellular glutamine to α-ketoglutarate ratio, which increases under conditions of nitrogen sufficiency (high concentration of ammonium ion) and decreases under conditions of nitrogen starvation (the presence of other nitrogen sources or concentrations of ammonium ion lower than 2 mM) (Doucette *et al.*, 2011; Ninfa and Atkinson, 2000). We assumed that this might explain why glutamate did not show any effect on the phosphorylation of $EIIA^{Ntr}$(K75D) and tryptophan fluorescence quenching of EI^{Ntr} while α-ketoglutarate and glutamine did (Figs. 3 and 6), although these three amino acids can readily exchange amino groups by glutamate dehydrogenase, glutamine synthetase and glutamate synthase in *E. coli* and other bacteria. We showed above that the phosphorylated form of $EIIA^{Ntr}$ significantly increased in *E. coli* cells supplemented with 20 mM alanine in W salts medium compared with cells supplemented with 20 mM ammonium salt (left panel of Fig. 2B). To further correlate the phosphorylation state of $EIIA^{Ntr}$ with the ratio of α-ketoglutarate to glutamine concentrations in the cell, we tested another condition known to increase this ratio for the effect on the *in vivo* phosphorylation state of the PTS^{Ntr}. As expected from the *in vitro* studies (Figs. 3–6), the phosphorylated form of $EIIA^{Ntr}$ significantly increased when the culture was subjected to a nitrogen downshift from 20 to 1 mM ammonium salt (right panel of Fig. 2B). The western blots suggest that the total amount of $EIIA^{Ntr}$ is comparable under both growth conditions.

To investigate the direct effect of α-ketoglutarate and glutamine on the phosphorylation state of the PTS^{Ntr} *in vivo*, we monitored changes of the phosphorylation state of $EIIA^{Ntr}$(K75D) dependent on the addition of α-ketoglutarate or glutamine to growth medium. The inclusion of 10 mM glutamine in W salts medium containing 20 mM alanine significantly increased the dephosphorylated form of $EIIA^{Ntr}$, while the inclusion of 10 mM α-ketoglutarate showed the opposite effect to a small degree (Fig. 8A). One possibility for this result could be that the intracellular level of α-ketoglutarate in cells grown in the medium containing 20 mM alanine is already high enough to produce full activation of the autophosphorylation of EI^{Ntr} in the cell. To investigate this possibility, we tested the effect of α-ketoglutarate in cells grown under a condition where the ratio of α-ketoglutarate to glutamine is low. However, the addition of 20 mM α-ketoglutarate in the medium supplemented with 20 mM ammonium salt had little effect on the phosphorylation state of $EIIA^{Ntr}$, ruling out this possibility (Fig. S5). Another possibility is that α-ketoglutarate is not permeable enough to support its accumulation in the cytoplasm. Dimethyl-α-ketoglutarate (dm-α-KG), a membrane-permeable ester known to be cleaved by cellular esterases to form

α-ketoglutarate in the cell, is often used to increase the intracellular level of α-ketoglutarate (Doucette *et al.*, 2011). The addition of 20 mM dm-α-KG to medium supplemented with 20 mM ammonium salt significantly increased the phosphorylated form of $EIIA^{Ntr}$ in cells (left panel of Fig. 8B), supporting the notion that a high ratio of α-ketoglutarate to glutamine stimulates the phosphorylation of the PTS^{Ntr}.

To test whether the GAF domain is necessary to sense nitrogen availability *in vivo*, we constructed a strain (KM201) deleted for both the *ptsN* gene (encoding $EIIA^{Ntr}$) and that encoding the N-terminal 169 amino acids covering the GAF domain in the *ptsP* gene (encoding EI^{Ntr}). Expression of the EI^{Ntr}(ΔGAF) protein was confirmed by Western blotting with an antibody raised against EI^{Ntr} (Fig. S6) and from the fact that $EIIA^{Ntr}$(K75D) could be phosphorylated in the KM201 strain harboring the pCR3(K75D) plasmid whereas it was not phosphorylated in the *ptsP* deletion mutant (CR103) harboring pCR3(K75D) (compare Figs. 2A and 8C). The data in Fig. 8C show that the phosphorylation state of $EIIA^{Ntr}$(K75D) is not influenced by the nitrogen sources in KM201/pCR3(K75D) cells. Furthermore, the effect of dm-α-KG was also dependent on the presence of the GAF domain (right panel of Fig. 8B), indicating the requirement of the GAF domain of EI^{Ntr} for sensing nitrogen availability *in vivo*.

We further investigated how the phosphorylation state of $EIIA^{Ntr}$ changes when cells are moved from an ammonium-rich environment to nitrogen depletion. CR301 cells harboring pCR3(K75D) were grown in W salts medium with 0.2% glucose as carbon source and 20 mM ammonium ion as nitrogen source to mid-logarithmic phase, at which point the culture was subjected to nitrogen depletion by moving cells to the same medium lacking the nitrogen source. It is well-known that α-ketoglutarate concentration in *E. coli* is rapidly elevated within a few minutes of nitrogen depletion with a concomitant decrease by an order of magnitude in the glutamine level. We monitored the phosphorylation state of $EIIA^{Ntr}$ upon nitrogen depletion over the course of 20 minutes. While $EIIA^{Ntr}$ exists mainly in a dephosphorylated form in cells growing in ammonia-rich medium, $EIIA^{Ntr}$ was about 70% phosphorylated in 5 minutes and it was almost completely phosphorylated in 10 minutes after cells were shifted to a medium depleted of nitrogen source (Fig. 8D). These data support the model that glutamine and α-ketoglutarate together control the phosphorylation state of the PTS^{Ntr} in response to nitrogen availability *in vivo*.

In our previous study (Lee *et al.*, 2007), we demonstrated that the dephosphorylated form of $EIIA^{Ntr}$ inhibited the Trk K^+ transport system by forming a tight complex with TrkA resulting in a decrease of the intracellular concentrations of K^+. Therefore, we determined whether the nitrogen source can influence intracellular K^+ levels. As shown in Fig. 9, intracellular K^+ levels in cells grown in the presence of 20 mM $(NH_4)_2SO_4$ were about 30% lower than those grown in the presence of 20 mM alanine as the sole nitrogen source, while K^+ levels were not affected by nitrogen sources in cells deleted for the GAF domain of EI^{Ntr}. These data suggest that glutamine and α-ketoglutarate modulate cellular potassium levels by regulating the phosphorylation state of the PTS^{Ntr} and that the GAF domain of EI^{Ntr} is essential for this regulation.

We previously indicated that some *E. coli* K-12 strains such as MG1655 are sensitive to leucine-containing peptides (LCP) due to a frameshift mutation in *ilvG* encoding acetohydroxy acid synthase (AHAS) II and have shown that a *ptsN* mutant is extremely sensitive whereas *ptsP* and *ptsO* mutants are more resistant than wild type MG1655 cells to LCPs (Lee *et al.*, 2005). In *E. coli*, there are three isozymes of AHAS, which catalyze the first common step in the biosynthetic pathway of the three branched-chain amino acids. While AHAS I and III are sensitive to valine, AHAS II is valine-resistant. Because AHAS II is not expressed in some *E. coli* K-12 strains, these strains are extremely sensitive to valine

(Lawther *et al.*, 1981) and, albeit to a less extent, to LCPs (Gollop *et al.*, 1982; Lee *et al.*, 2005). Since LCPs in the medium induce the accumulation of abnormally high cellular concentrations of leucine for an unknown reason, biosynthesis of isoleucine and valine is feedback-inhibited in *ilvG* mutant cells; therefore, growth of *E. coli* MG1655 cells is retarded in the presence of LCPs. The extreme sensitivity of a *ptsN* mutant of *E. coli* MG1655 to LCPs was shown to be due to a further decrease in both the level and total activity of AHAS caused by the increase in cellular potassium; dephosphorylated $EIIA^{Ntr}$ was shown to be required to neutralize the sensitivity of *E. coli* K12 strains to LCPs (Lee *et al.*, 2007). Therefore, we compared the KM101 strain, deleted for the GAF domain of EI^{Ntr}, with wild-type MG1655 for sensitivity to the Ala-Leu dipeptide in the presence of different nitrogen sources, based on the length of the lag period for the growth of cultures. While the wild-type strain was more resistant to Ala-Leu in a nitrogen-rich medium than in a nitrogen-limited medium, the sensitivity of KM101 cells to Ala-Leu was not influenced by nitrogen sources; KM101 cells were equally sensitive to Ala-Leu in both nitrogen sources (Fig. 10). This result further supports the thesis that the GAF domain is necessary to sense nitrogen availability *in vivo*.

Sensing of nitrogen availability by the PTS^{Ntr} is independent of the ilvG genotype

Besides the sensitivity to leucine or LCPs, a growth defect of the *ptsN* mutant on certain organic nitrogen sources was recently shown to be observed only in *E. coli* strains lacking a functional *ilvG* gene (Reaves and Rabinowitz, 2011). Therefore, involvement of the PTS^{Ntr} in nitrogen regulation has been challenged (Ninfa, 2011). To clarify whether the effect of the ratio of glutamine to α-ketoglutarate on the PTS^{Ntr} activity is dependent on the *ilvG* genotype *in vivo*, we constructed an $ilvG^{+}$ revertant of the *E. coli* MG1655 strain and tested effects of nitrogen sources on the phosphorylation state of the PTS^{Ntr} in cells. As shown in Fig. 8E, the dephosphorylated form of $EIIA^{Ntr}$ prevails in *E. coli* MG1655 $ilvG^{+}$ cells supplemented with 20 mM ammonium sulfate, whereas exposure of these cells to the same concentration of alanine favors the phosphorylated state. These data show that the effect of glutamine and α-ketoglutarate on the phosphorylation state of the PTS^{Ntr} is independent of the *ilvG* genotype.

In aggregate, the studies presented here make a strong case that glutamine and α–ketoglutarate levels, as the canonical signals of nitrogen limitation, determine the state of phosphorylation of $EIIA^{Ntr}$ and that the mediator of the regulation is the GAF domain of EI^{Ntr}.

Discussion

The work described here required the development of a method to evaluate the state of phosphorylation of $EIIA^{Ntr}$. While the wild-type form of $EIIA^{Glc}$ exhibits a PDMS, that of $EIIA^{Ntr}$ does not (Fig. 1A). To search for the appropriate modification of $EIIA^{Ntr}$ that would result in a PDMS, systematic site-directed mutagenesis was carried out. The creation (K75D mutant) of a form of $EIIA^{Ntr}$ exhibiting both physiological activity (Fig. 1B) and a PDMS (Fig. 1A) provided the foundation for defining the mechanism by which the *in vivo* phosphorylation state of $EIIA^{Ntr}$ is regulated.

The measurement of the *in vivo* state of phosphorylation (Figs. 2 and 8) of $EIIA^{Ntr}$(K75D) was consistent with the *in vitro* results (Figs. 3 and 4). The presence of glutamine increased while α–ketoglutarate decreased the level of the dephosphorylated form of $EIIA^{Ntr}$(K75D). The overall results from both *in vitro* and *in vivo* tests are consistent with the model that glutamine and α–ketoglutarate serve as regulatory signals of the nitrogen PTS.

Rabus *et al.* previously searched for specific regulators of EI^{Ntr} (Rabus *et al.*, 1999), but effects of glutamine and α-ketoglutarate were not observed. Major differences between their and our current studies are that we used EI^{Ntr} that was purified as a soluble form without denaturation as well as a direct protein phosphorylation assay while they used a protein solubilized with urea, then renatured, and an indirect mannitol phosphorylation assay.

It is interesting to note that, while EI^{Ntr} autophosphorylation is stimulated by α-ketoglutarate (this study), the opposite effect (inhibition by α-ketoglutarate) appears to operate with the enzyme I of the sugar PTS (EI^{sugar}) (Doucette *et al.*, 2011). The present data demonstrate that the interaction of α-ketoglutarate with EI^{Ntr} requires the presence of the GAF domain present in EI^{Ntr} but not in EI^{sugar}. The explanation for the GAF domain-independent interaction with EI^{sugar} remains to be elucidated.

Despite the finding that the "nitrogen" PTS encoding NPr and $EIIA^{Ntr}$ was located in the same operon with *rpoN* encoding the nitrogen-related sigma factor and the observation of a growth defect of *ptsN* mutants on certain poor nitrogen sources (Powell *et al.*, 1995), there has been little evidence that any component of the so-called PTS^{Ntr} is directly involved in nitrogen metabolism or influences the genetic regulatory system for nitrogen assimilation, the *bona fide* Ntr system. In the present work, the phosphorylation state of the nitrogen PTS was shown to be regulated by glutamine and α-ketoglutarate, the canonical signals of nitrogen metabolism. Autophosphorylation of EI^{Ntr} was inhibited by glutamine and activated by α-ketoglutarate.

This regulatory mechanism is somewhat similar to that of the *bona fide* Ntr system, the genetic regulatory system for the activity of glutamine synthetase and transcription of other nitrogen-related genes; glutamine inhibits the uridylyltransferase activity of GlnD and the adenylyl-removing activity of GlnE and activates the uridylyl-removing activity of GlnD and the adenylyltransferase activity of GlnE to decrease nitrogen-regulated (Ntr) gene expression, whereas α-ketoglutarate exerts the opposite effects. α-Ketoglutarate also directly binds to PII and regulates the ability of PII to interact with GlnE and NRII to increase Ntr gene expression (Jiang *et al.*, 1998; Jiang *et al.*, 2007; Kamberov *et al.*, 1995; Ninfa and Jiang, 2005). Our demonstration of the role of glutamine and α-ketoglutarate in regulating PTS^{Ntr} provides an important link to the regulation of nitrogen metabolism.

The finding that the regulatory effects of glutamine and α-ketoglutarate are mediated by EI^{Ntr} places the locus of the regulatory signals at the first step of the nitrogen PTS pathway. The PTS^{Ntr} is highly conserved in all proteobacterial branches except for the ε-subdivision. A major structural difference between EI and EI^{Ntr} is the presence of the GAF domain in EI^{Ntr}. Until now, the function of the GAF domain in EI^{Ntr} has been elusive. The finding that the regulatory effects of glutamine and α-ketoglutarate require the presence of the GAF domain has now explained the rationale for its presence in EI^{Ntr}. GAF domains were initially found in cGMP phosphodiesterases, *Anabaena* adenylate cyclases, and *E. coli* FhlA and have been shown to be important for signal perception. The GAF domain (N-terminal 127 amino acids) of EI^{Ntr} exhibits a homology throughout its length to the N-terminal GAF domains of NifA proteins of the free-living diazotrophs with 21–26% identity (Reizer *et al.*, 1996) and displays a slightly less homology to sodium-responsive GAF domains in *Anabaena* adenylate cyclases, the formate-responsive GAF domain of *E. coli* FhlA, and cGMP phosphodiesterases. Intriguingly, the GAF domain of the transcriptional activator NifA was previously shown to directly bind α-ketoglutarate to resist inhibition by NifL under nitrogen-limiting condition in *Azotobacter vinelandii*, although the effect of glutamine was not tested (Little and Dixon, 2003). Therefore, binding of glutamine and α-ketoglutarate to GAF domains of EI^{Ntr} and thus regulation of the phosphorylation of the PTS^{Ntr} in response to nitrogen availability might be widely conserved among proteobacteria.

Experimental Procedures

Bacterial strains, plasmids, and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 1. All plasmids were constructed using standard PCR-based cloning procedures and verified by sequencing. Bacterial cells were grown as described previously (Lee *et al.*, 2010). *Escherichia coli* MG1655 derivatives with deletions of the GAF domain (residues 1–169) of EI^{Ntr} were constructed using the lambda red recombination method (Datsenko and Wanner, 2000). The kanamycin resistance (Km^R) cassette from plasmid pKD13 was amplified using the following primers: forward primer, 5'- ACA CCA GGT GCT GCC GGT AAT GCG CGG ATT CG**C AT**C GCT TGG CGA TAT TGG TGT AGG CTG GCG CTG CTT-3' and reverse primer, 5'- CAC AAA ACG CAT CTG CTT ATC GAC GTA AAA GAG GTT AAG TCA CGC CAA TTA TTC CGG GGA TCC GTC GAC C-3'. A new ATG start codon was inserted in the forward primer (in boldface type) to express a truncated EI^{Ntr} protein.

ilvG$^+$ revertants of *E. coli* MG1655 were generated using the lambda red recombination method (Datsenko and Wanner, 2000). The functional *ilvG* gene was amplified from an *E. coli* BL21 strain by PCR and introduced into *E. coli* MG1655 harboring pKD46. *E. coli* MG1655 *ilvG*$^+$ revertants were selected based on valine resistance by growing on M9 medium supplemented with 0.5% glucose and 0.2 mM valine. After the plasmid pKD46 was cured, the *ilvG*$^+$ genotype was confirmed by DNA sequencing.

Purification of overexpressed proteins

Purification of soluble His-tagged proteins (His-EI, His-HPr, His-$EIIA^{Glc}$, EI^{Ntr}-His, His-EI^{Ntr}(ΔGAF), His-$EIIA^{Ntr}$ and His-$EIIA^{Ntr}$(K75D) was accomplished as described previously (Lee *et al.*, 2005), with slight modifications. *E. coli* GI698 harboring specific plasmids were grown and protein expression was induced as described previously for overproduction (LaVallie *et al.*, 1993). His-tagged proteins were purified using BD TALON™ metal affinity resin (BD Biosciences Clontech) according to the manufacturer's instructions and bound proteins were eluted with binding buffer containing 200 mM imidazole. The fractions containing His-tagged proteins were pooled and concentrated in a 3K Macrosep centrifugal concentrator (Pall Gelman Laboratory). To obtain homogeneous proteins (>98% pure) and to remove imidazole, the concentrated pool was chromatographed on a HiLoad 16/60 Superdex 75 prepgrade column (GE Healthcare Life Sciences) equilibrated with buffer A (20 mM HEPES-KOH, pH 8.0 containing 200 mM NaCl). Note that purified EI^{Ntr} containing the GAF domain has a tendency to aggregate (Piszczek *et al.*, 2011). Therefore, meaningful studies of the properties of this protein are limited to relatively dilute solutions.

Purification of insoluble His-tagged proteins (His-$EIIA^{Ntr}$(I69D), His-$EIIA^{Ntr}$(A70D), His-$EIIA^{Ntr}$(I71D), His-$EIIA^{Ntr}$(P72D), His-$EIIA^{Ntr}$(G74D) and His-$EIIA^{Ntr}$(L76D)) was accomplished using 6 M urea as described previously (Kim *et al.*, 2011) with some modifications. After disruption of cells, overexpressed proteins in inclusion bodies were solubilized with 6 M urea and centrifuged. The supernatant solution was mixed with 500 μl TALON metal affinity resin (BD Biosciences Clontech) and agitated for 20 min at 4 °C. The resin was then centrifuged and washed sequentially with 5 volumes of Buffer A containing 3, 1.5, 0.75, and 0 M urea. Proteins were then eluted with 1 volume of Elution Buffer (20 mM HEPES-KOH containing 300 mM NaCl and 200 mM imidazole, pH 8.0). The concentrated pool was chromatographed on a HiLoad 16/60 Superdex 75 prepgrade column to remove imidazole and any remaining urea.

In vitro phosphorylation assays

All reactions were performed with purified proteins in the presence of 1 mM PEP, 0.1 M Tris00B7HCl, pH 7.5, 2 mM $MgCl_2$, 1 mM EDTA,10 mM KCl, 0.5 mM dithiothreitol in a total volume of 20 μl. To measure a PDMS of $EIIA^{Glc}$, $EIIA^{Glc}$ (2 μg) was incubated with EI (1 μg) and HPr (1 μg) and to measure a PDMS of $EIIA^{Ntr}$, $EIIA^{Ntr}$ (2 μg) was incubated with EI^{Ntr} (1 μg) and NPr (1 μg). After incubation at 37 °C for 10 min, reactions were stopped by the addition of 5 μl of SDS-PAGE sample buffer (250 mM Tris·Cl, pH 6.8, 10% glycerol, 1% SDS, 150 mM 2-mercaptoethanol) and then analyzed by SDS PAGE. The proteins were stained with Coomassie Brilliant Blue.

To examine the effect of various metabolites on the phosphotransferase activity of the PTS^{Ntr}, $EIIA^{Ntr}$(K75D) (3 μg) was incubated with trace amounts of EI^{Ntr} or EI^{Ntr}(ΔGAF) and NPr in the presence of each metabolite tested (5 mM). After incubation for the indicated times at room temperature, reactions were stopped by the addition of 5 μl of SDS-PAGE sample buffer and analyzed by 14% SDS-PAGE. The proteins were stained with Coomassie Brilliant Blue.

To test the effect of metabolites on the autophosphorylation of EI^{Ntr} and EI^{Ntr}(ΔGAF), 0.5 μg of purified proteins were incubated with [^{32}P]PEP (20 μM) and various concentrations of metabolites. After incubation for 1 min at room temperature, reactions were stopped by the addition of 5 μl of SDS-PAGE sample buffer, and then analyzed by SDS-PAGE (4–20% gradient gel). The labeled proteins were visualized by autoradiography.

Measurement of the *in vivo* phosphorylation state of $EIIA^{Ntr}$

To determine the *in vivo* phosphorylation state of $EIIA^{Ntr}$, we made polyclonal antibodies against $EIIA^{Ntr}$ using female ICR mice. The CR301 and KM201 cells harboring the expression vector pCR3(K75D) (See Table 1) were grown in W salts medium containing 20 mM $(NH_4)_2SO_4$ and 0.2% glucose to mid-logarithmic phase. Cells were harvested, washed and resuspended in W salts medium containing 0.2% glucose with different nitrogen sources as indicated in each figure and legend. After growth at 37 °C to A_{600} = 0.8 or mid-logarithmic phase, an aliquot (0.2 ml) of the cell culture was quenched to stabilize the phosphorylation state of the PTS^{Ntr} components by adding 20 μl of 10 M NaOH followed by vortexing for 10 s, and then 180 μl of 3 M sodium acetate (pH 5.2) and 1 ml of ethanol were added. Samples were chilled at −70 °C for at least 15 min, thawed and centrifuged at 4 °C. The pellet was rinsed with 70% ethanol and resuspended in 100 μl of SDS sample buffer, and 20 μl of this solution was run on SDS–PAGE (15% gel). Proteins were then electrotransferred onto immobilin-P (Millipore, MA) following the manufacturer's protocol and were detected by immunoblotting using antiserum against $EIIA^{Ntr}$.

Intrinsic tryptophan fluorescence measurements

Fluorescence measurements were carried out with a CARY Eclipse fluorescence spectrophotometer with excitation at 280 nm at room temperature. The spectral bandwidths were 5 and 10 nm, respectively for excitation and emission. The emission spectrum was monitored between 300 and 400 nm. EI^{Ntr} and EI^{Ntr}(ΔGAF) were present at 95 μg/ml in 50 mM Tris (pH 7.5) containing 20 mM NaCl. For determination of the respective *K*d values, various concentrations of metabolites (0.5–32 mM) were examined for the degree of fluorescence quenching.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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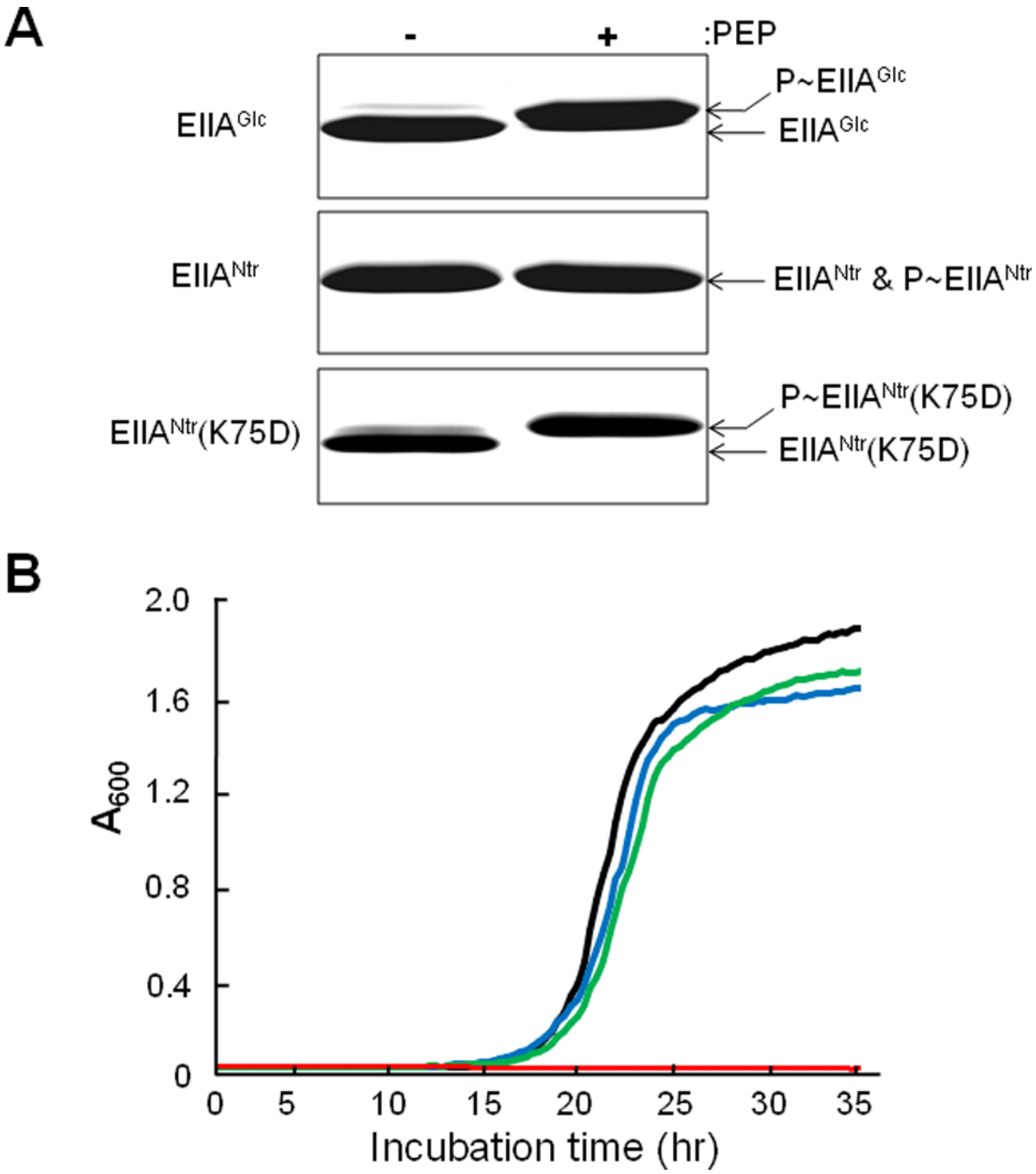

Fig. 1.

An EIIA^{Ntr} mutant exhibits a PDMS on SDS-PAGE and neutralizes growth inhibition by Ala-Leu.

A. EIIA^{Glc}, EIIA^{Ntr} and EIIA^{Ntr}(K75D) were incubated under phosphorylating conditions with and without 1 mM PEP (see Experimental Procedures) and then analyzed by SDS-PAGE.

B. Cells grown in LB medium overnight were washed with M9 medium, inoculated into M9 medium containing 0.5% glucose supplemented with 0.5 mM Ala-Leu, and growth at 37 °C was recorded by measuring the optical density at 600 nm: black line, MG1655; red, CR301(Δ*ptsN*); blue, CR301/pCR3(IIA^{Ntr}); and green, CR301/pCR3(K75D).

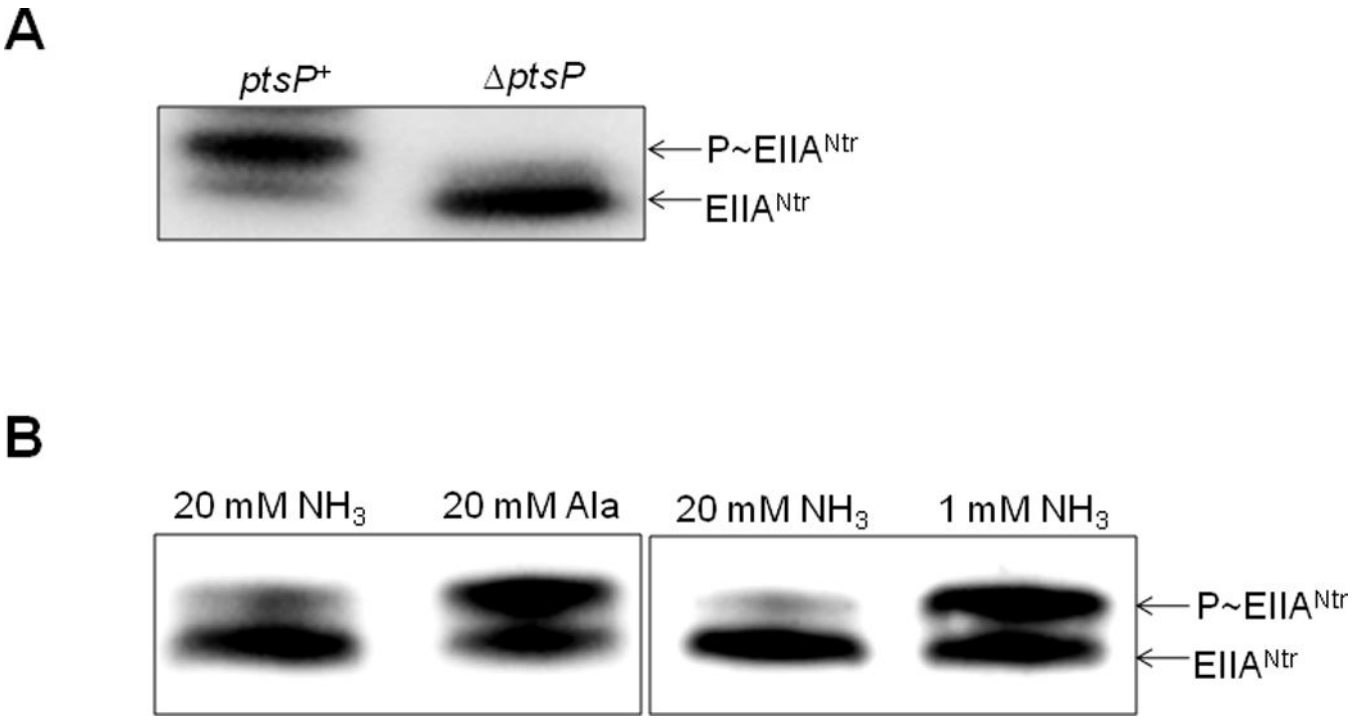

Fig. 2.

Measurement of the *in vivo* phosphorylation state of $EIIA^{Ntr}$ under various conditions. The phosphorylation state of $EIIA^{Ntr}$ was determined in cells harboring the pCR3(K75D) plasmid expressing $EIIA^{Ntr}$(K75D) by Western blotting as described in Experimental Procedures.

A. Determination of the phosphorylation state of $EIIA^{Ntr}$ in CR301/pCR3(K75D) and CR103/pCR3(K75D) cells grown in LB medium to A_{600} = 2.0.

B. CR301/pCR3(K75D) cells were grown in W salts medium containing 20 mM $(NH_4)_2SO_4$ and 0.2% glucose to mid-logarithmic phase, then centrifuged, washed and resuspended in W salts medium containing 0.2% glucose with 20 mM $(NH_4)_2SO_4$, 20 mM alanine, or 1 mM $(NH_4)_2SO_4$. When the A_{600} reached 0.8, intracellular phosphorylation states were determined.

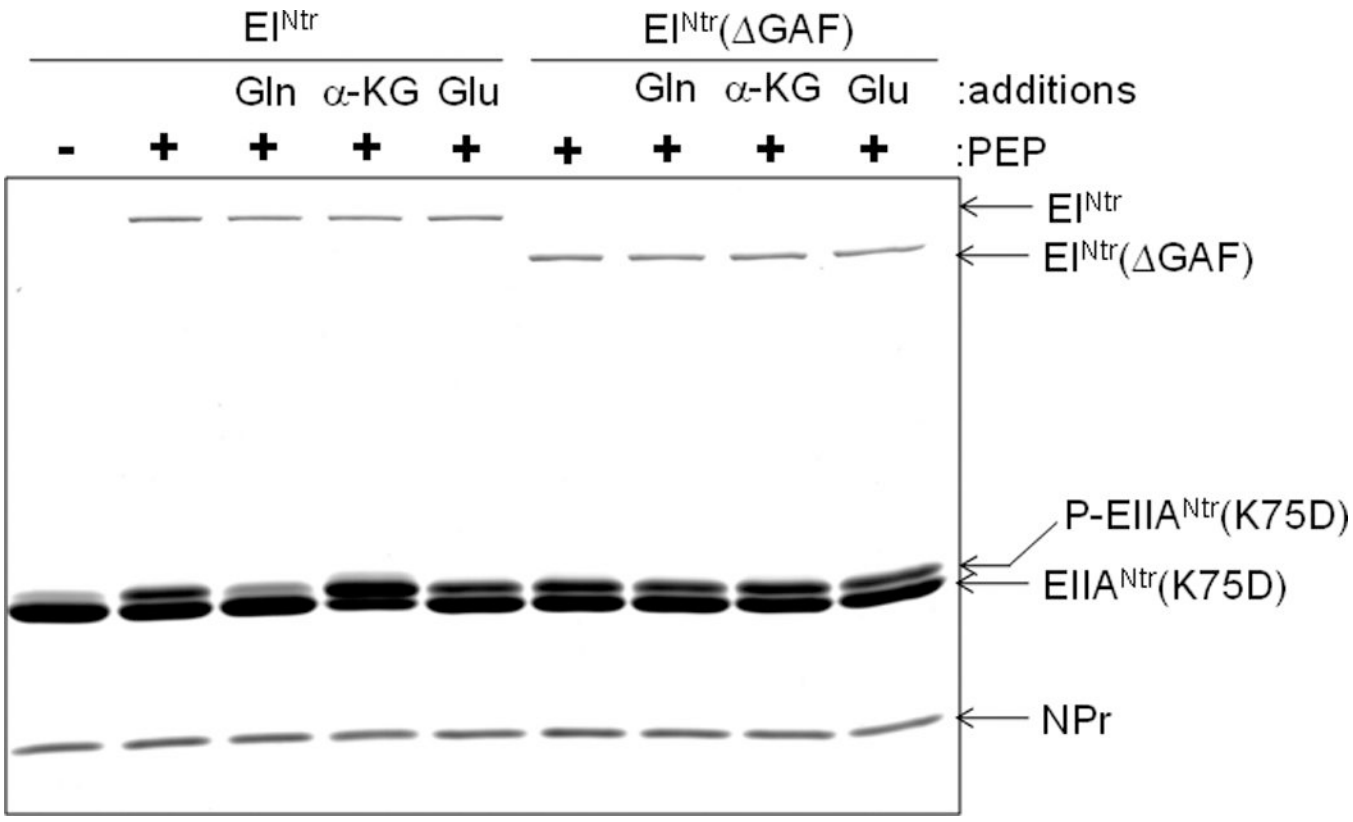

Fig. 3.

Effect of glutamine and α–ketoglutarate on the phosphorylation of EIIA^{Ntr}(K75D). EIIA^{Ntr}(K75D) (3 μg) was incubated with PEP (1 mM), EI^{Ntr} or EI^{Ntr}(ΔGAF) (0.1 μg) and NPr (0.3 μg) in the presence of glutamate (Glu), glutamine (Gln) or α-ketoglutarate (α-KG) at 5 mM. After incubation for 1 min at room temperature, reactions were stopped by the addition of SDS-PAGE sample buffer and analyzed by 14% SDS-PAGE. The proteins were stained with Coomassie Brilliant Blue.

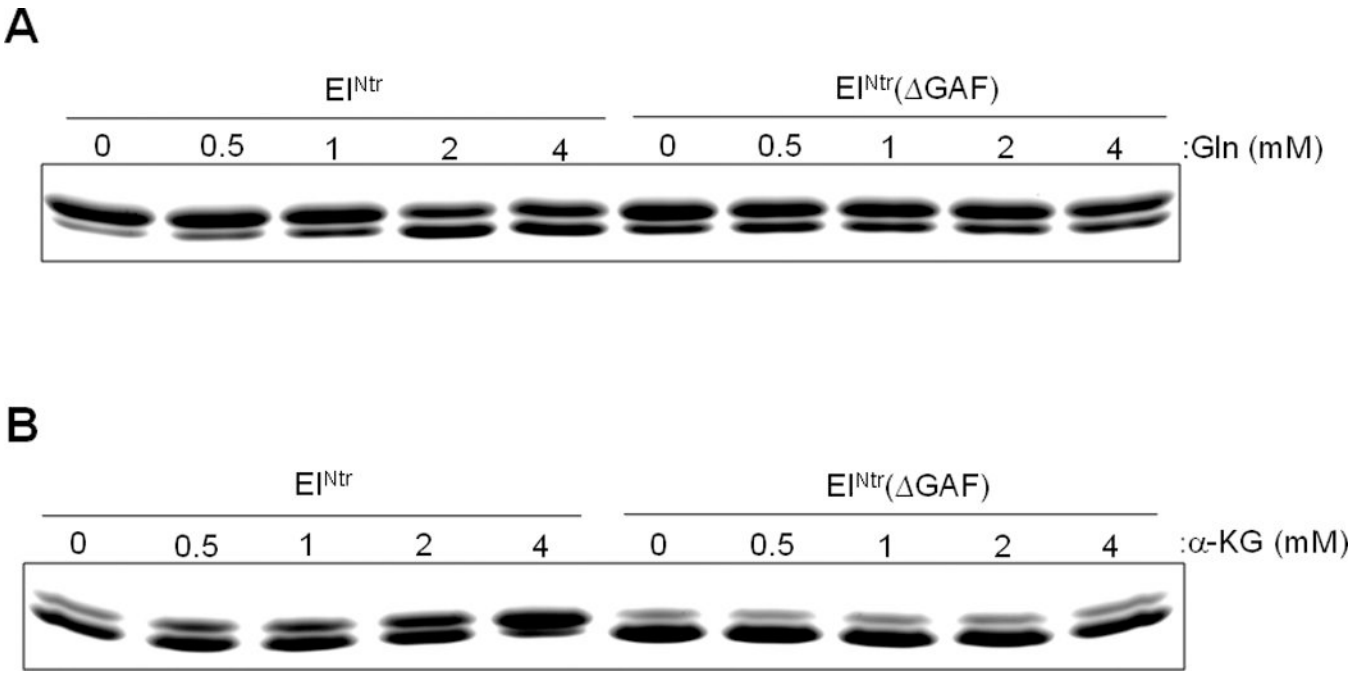

Fig. 4.

Glutamine and α–ketoglutarate affect the phosphorylation of EIIA^{Ntr}(K75D) in a dose-dependent manner. *In vitro* phosphorylation reactions were carried out as described in Figure 3 in the presence of purified EI^{Ntr} or EI^{Ntr}(ΔGAF), NPr and EIIA^{Ntr}(K75D) with PEP (1 mM) and the indicated concentrations of glutamine (A) or α–ketoglutarate (B). After incubation at room temperature for 2 min (A) or 0.5 min (B), reactions were stopped by the addition of SDS-PAGE sample buffer and then run on a 14% polyacrylamide gel under denaturing conditions.

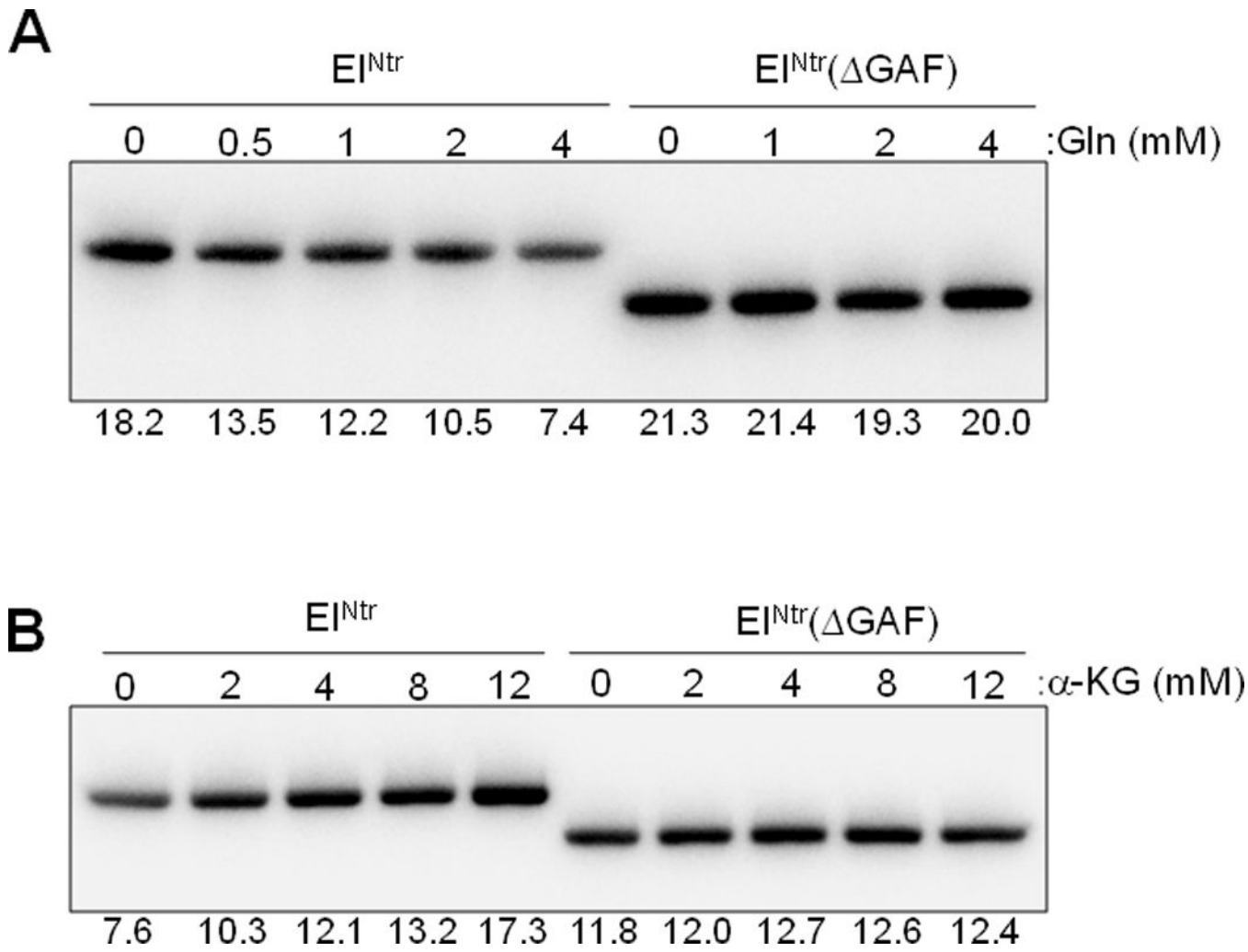

Fig. 5.

The autophosphorylation of EI^{Ntr} is affected by glutamine and α–ketoglutarate. *In vitro* phosphorylation reactions were carried out in the presence of purified EI^{Ntr} or EI^{Ntr}(ΔGAF) and [^{32}P]PEP (20 μM) with the indicated concentrations of glutamine (A) or α–ketoglutarate (B) at room temperature for 2 min (A) or 1 min (B) and run on a 14% polyacrylamide gel under denaturing conditions. The labeled proteins were visualized by autoradiography. Band intensities were quantified using Multi Gauge V3.0 software and relative band intensities are shown under each panel.

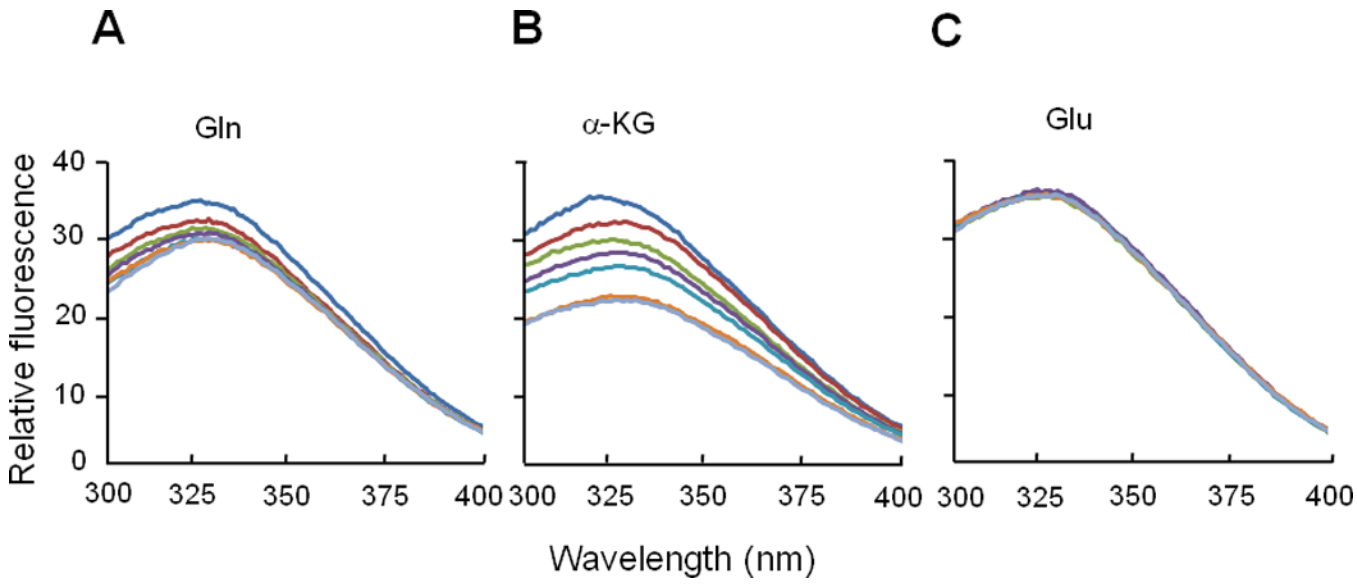

Fig. 6.

Effect of glutamine, α-ketoglutarate and glutamate on tryptophan fluorescence of EI^{Ntr}. Tryptophan fluorescence was measured as described in "Experimental Procedures". EI^{Ntr} was present at 95 μg/ml in 50 mM Tris, pH 7.5, 20 mM NaCl. For determination of the respective *K*d values, a series of ligand concentrations were examined for the degree of quenching of fluorescence: blue, control; brown, 0.5 mM; yellow-green, 1 mM; purple, 2 mM; sky-blue, 4 mM; orange, 16 mM; gray, 32 mM.

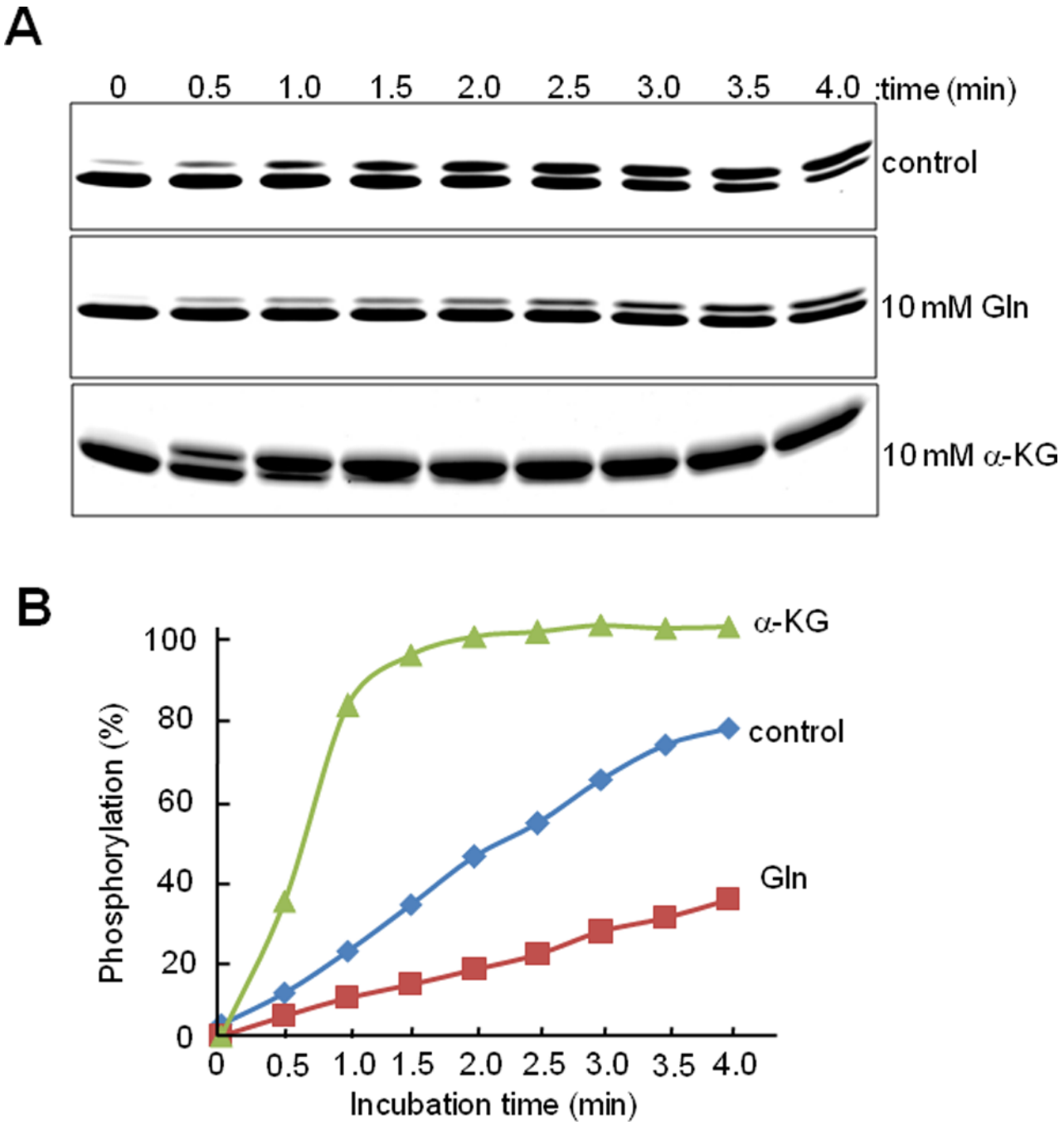

Fig. 7.

Effect of glutamine and α–ketoglutarate on the phosphorylation of EIIA^{Ntr}(K75D) as a function of time.

A. EIIA^{Ntr}(K75D) (3 μg) was incubated with PEP (1 mM), EI^{Ntr} (0.05 μg) and NPr (0.2 μg) in the presence of glutamine (Gln) or α-ketoglutarate (α-KG) at 10 mM. After incubation for the indicated time periods at room temperature, reactions were stopped by the addition of SDS-PAGE sample buffer and analyzed by 14% SDS-PAGE. The proteins were stained with Coomassie Brilliant Blue.

B. The band intensities in panel A were quantified using the Multi Gauge V3.0 software and the levels of the phosphorylated form of $EIIA^{Ntr}$(K75D) were plotted as the percent of total $EIIA^{Ntr}$(K75D).

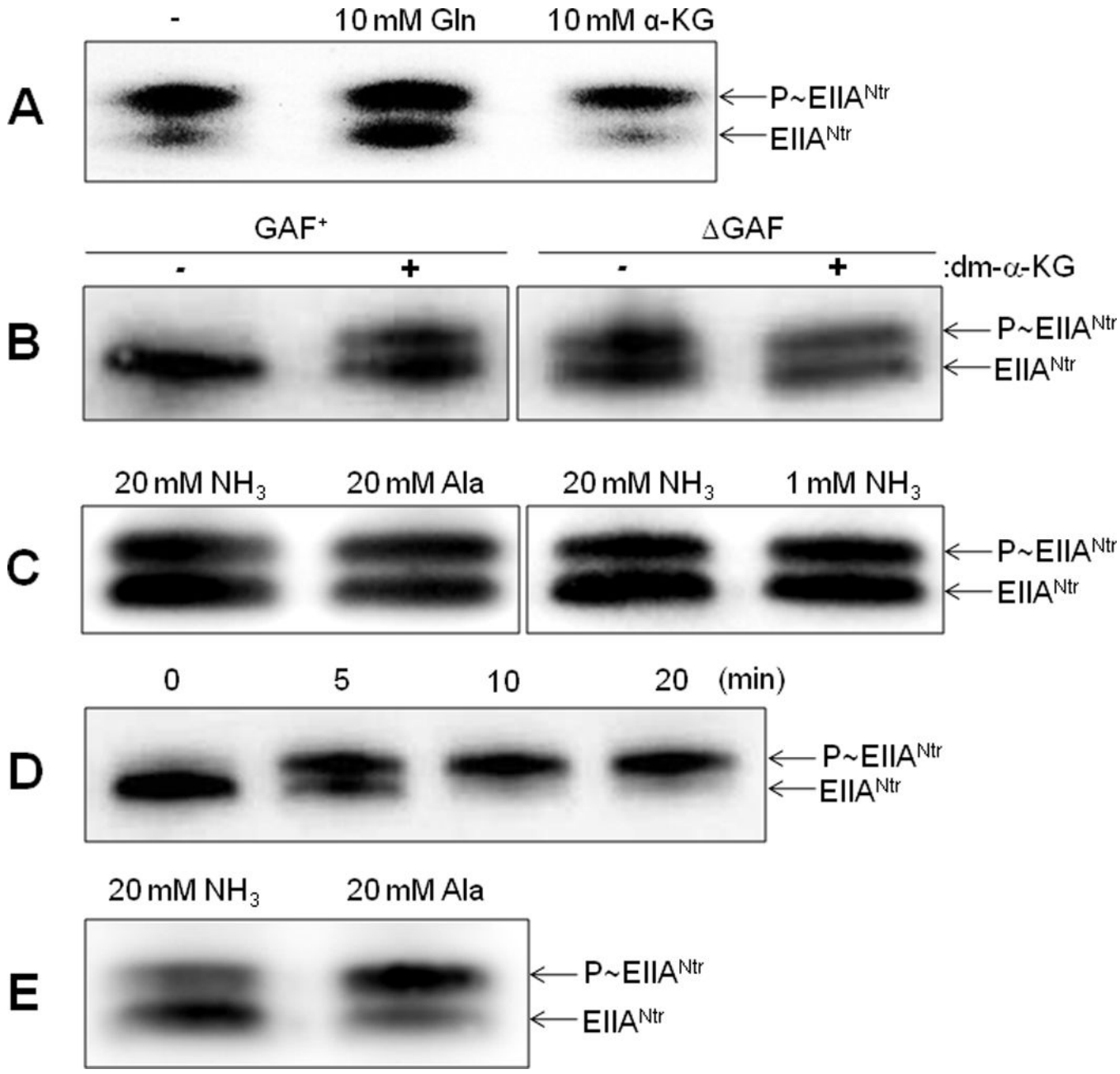

Fig. 8.

Metabolite effects on the *in vivo* phosphorylation state of EIIA^{Ntr}. The intracellular phosphorylation state of EIIA^{Ntr}(K75D) was determined in each strain as described in Experimental Procedures.

A. CR301 cells harboring the pCR3(K75D) plasmid expressing EIIA^{Ntr}(K75D) were grown in W salts medium containing 20 mM alanine in the presence of 10 mM glutamine or 10 mM α-ketoglutarate.

B. CR301 (left panel, GAF^{+}) and KM201 (right panel, ΔGAF) cells harboring the pCR3(K75D) plasmid were grown in W salts medium containing 20 mM $(NH_4)_2SO_4$ and 0.2% glucose in the presence and absence of 10 mM dimethyl-α-ketoglutarate (dm-α-KG).

C. KM201/pCR3(K75D) cells were grown in W salts medium containing 0.2% glucose with 20 mM $(NH_4)_2SO_4$, 20 mM alanine, or 1 mM $(NH_4)_2SO_4$. The intracellular phosphorylation state of $EIIA^{Ntr}$(K75D) was determined when A_{600} reached 0.8 in A–C.

D. Effect of nitrogen depeletion on the phosphorylation state of $EIIA^{Ntr}$. CR301/pCR3(K75D) cells were grown in W salts medium containing 20 mM $(NH_4)_2SO_4$ and 0.2% glucose to mid-logarithmic phase, then centrifuged, washed and resuspended in W salts medium containing 0.2% glucose with no nitrogen source. After incubation for the indicated time periods, intracellular phosphorylation states of $EIIA^{Ntr}$ were determined.

E. Effect of nitrogen availability on the PTS^{Ntr} activity in *ilvG*$^+$ *E. coli*. CR303 cells harboring the plasmid pCR3(K75D) were grown in W salts medium containing 20 mM $(NH_4)_2SO_4$ and 0.2% glucose to mid-logarithmic phase, then centrifuged, washed and resuspended in W salts medium containing 0.2% glucose with 20 mM $(NH_4)_2SO_4$ or 20 mM alanine. When A_{600} reached 0.8, the intracellular phosphorylation state of $EIIA^{Ntr}$(K75D) was determined as described in Experimental Procedures.

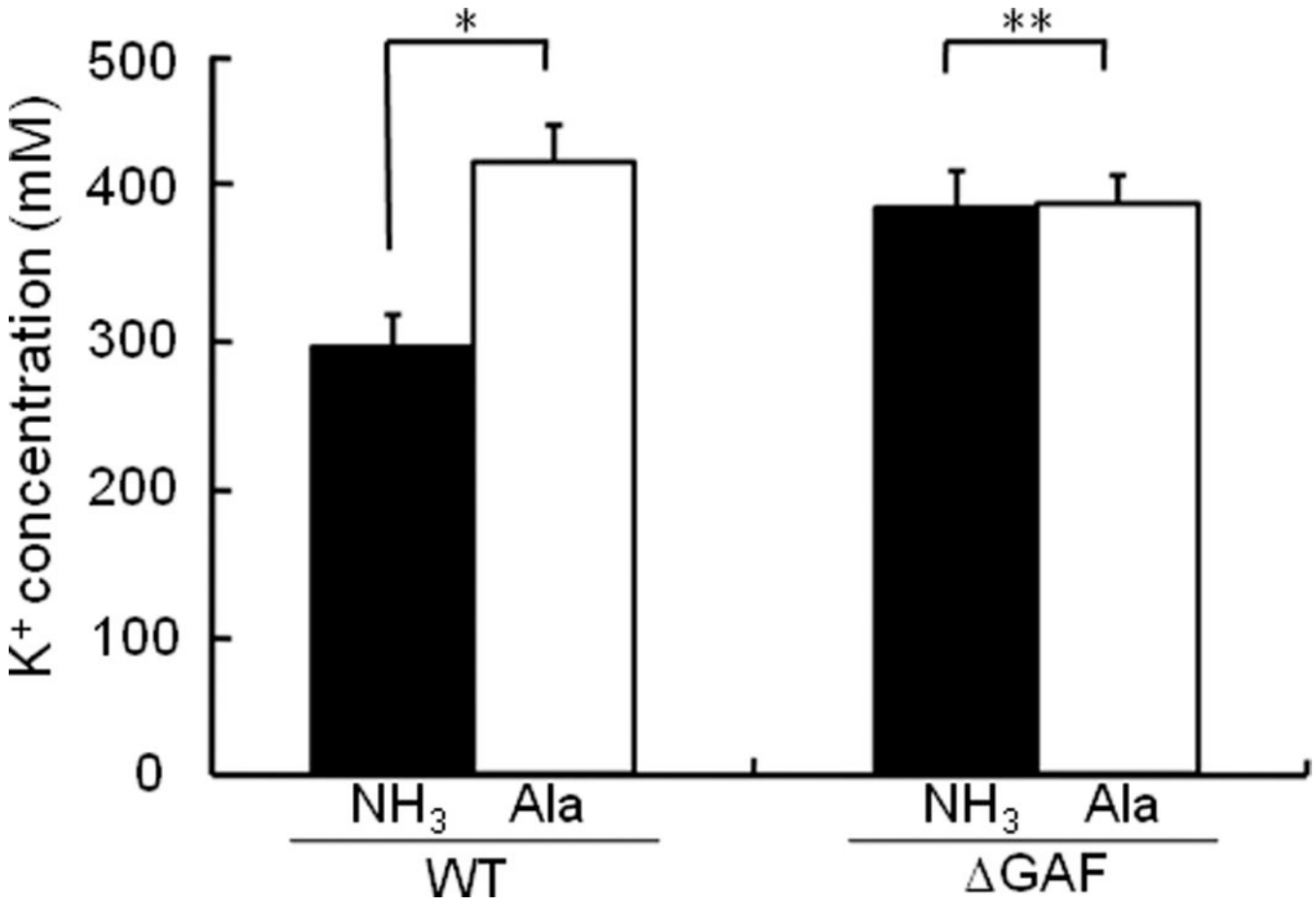

Fig. 9.

Effect of nitrogen source on the intracellular level of potassium. *E. coli* MG1655 (WT) and KM101 (ΔGAF) cells were grown in W salts medium supplemented with 20 mM $(NH_4)_2SO_4$ or alanine as the sole nitrogen source to A_{600} = 0.5 and intracellular K^+ concentrations were measured as described previously (Lee *et al.*, 2007). Each column represents the mean ± S.D. of 5 measurements and statistical significance of differences between groups was analyzed by Student's *t* test: *, $p < 0.001$; **, $p > 0.1$.

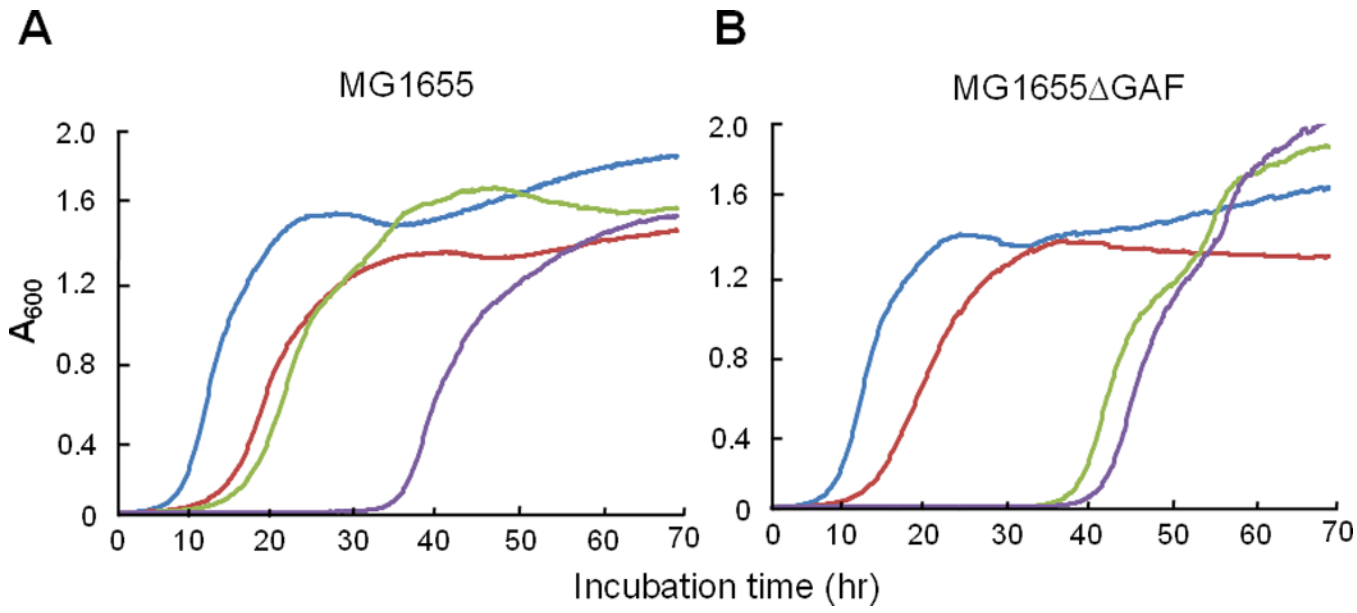

Fig. 10.

The GAF domain and nitrogen availability affect the sensitivity of *E. coli* MG1655 cells to Ala-Leu dipeptide. Wild-type (A) and KM101 (B) cells grown in LB medium overnight were washed with M9 medium, inoculated into W salts medium containing 0.5% glucose with 20 mM $(NH_4)_2SO_4$ (blue) or 20 mM alanine (red), and growth was recorded by measuring the optical density at 600 nm. To test the effect of a leucine-containing peptide, 0.5 mM Ala-Leu was added into W salts medium containing 0.5% glucose with 20 mM $(NH_4)_2SO_4$ (green) or 20 mM alanine (purple).

Table 1

Escherichia coli strains and plasmids used in this study.

Strain or plasmid	Genotype or phenotype	Source or Reference
Strains		
MG1655	F^- λ^- *ilvG*$^-$ *rfb-50 rph-1*. Wild type *E. coli* K-12	(Blattner *et al.*, 1997)
MG1655 *ilvG*$^+$	F^- λ^- *rfb-50 rph-1*	This study
GI698	F^- λ^- *lacI*^{q} *lacPL8 ampC*::P_{trp} cI	(LaVallie *et al.*, 1993)
CR103	MG1655 *ptsP::cat ptsN::*Tet^R	This study
CR301	MG1655 *ptsN::*Tet^R	(Lee *et al.*, 2005)
CR303	MG1655 *ilvG*$^+$ *ptsN::*Tet^R	This study
KM101	MG1655 ΔGAF	This study
KM201	MG1655 *ptsN::*Tet^R ΔGAF	This study
Plasmids		
pRE1	Expression vector under control of λP_L promoter, Amp^r	(Reddy *et al.*, 1989)
pCR3	pRE1-based expression vector for $EIIA^{Ntr}$	(Lee *et al.*, 2005)
pCR3(H73A)	pRE1-based expression vector for $EIIA^{Ntr}$(H73A)	(Lee *et al.*, 2005)
pCR3(K75D)	pRE1-based expression vector for $EIIA^{Ntr}$(K75D)	This study
pCR1H	pRE1-based expression vector for EI^{Ntr} with C-terminal 6 histidines	This study
pCR1H(ΔGAF)	pRE1-based expression vector for EI^{Ntr}(ΔGAF) with N-terminal 6 histidines	This study
pCR3H	pRE1-based expression vector for $EIIA^{Ntr}$ with N-terminal 6 histidines	This study
pCR3H(K75D)	pRE1-based expression vector for $EIIA^{Ntr}$(K75D) with N-terminal 6 histidines	This study
pCR3H(I69D)	pRE1-based expression vector for $EIIA^{Ntr}$(I69D) with N-terminal 6 histidines	This study
pCR3H(A70D)	pRE1-based expression vector for $EIIA^{Ntr}$(A70D) with N-terminal 6 histidines	This study
pCR3H(I71D)	pRE1-based expression vector for $EIIA^{Ntr}$(I71D) with N-terminal 6 histidines	This study
pCR3H(P72D)	pRE1-based expression vector for $EIIA^{Ntr}$(P72D) with N-terminal 6 histidines	This study
pCR3H(G74D)	pRE1-based expression vector for $EIIA^{Ntr}$(G74D) with N-terminal 6 histidines	This study
pCR3H(L76D)	pRE1-based expression vector for $EIIA^{Ntr}$(L76D) with N-terminal 6 histidines	This study
pPR3H(E86A)	pRE1-based expression vector for $EIIA^{Glc}$(E86A) with N-terminal 6 histidines	This study
pKD13	Template plasmid, Km^r Amp^r	(Datsenko and Wanner, 2000)
pKD46	Vector encoding arabinose-inducible λ-Red recombinase, Amp^r	(Datsenko and Wanner, 2000)