**The nitrogen regulatory PII protein (GlnB) and N-acetyl-glucosamine 6-phosphate epimerase (NanE) allosterically activate glucosamine 6-phosphate deaminase (NagB) in *Escherichia coli*.**

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**ABSTRACT**

Nitrogen is important for many cellular processes, and amino sugars are good sources of ammonia, produced by NagB, glucosamine 6-phosphate deaminase. NagB is known to be allosterically regulated by N-acetyl-glucosamine 6-phosphate (GlcNAc-6P) and the phosphocarrier protein of the bacterial phosphotransferase system, HPr, in *Escherichia coli*. We provide evidence that NanE, GlcNAc-6P epimerase, and the uridylylated PII protein allosterically activate NagB by direct protein-protein interactions. NanE is essential for neuraminic acid (NANA) and N-acetylmannosamine (ManNAc) utilization, and PII is known to be a central metabolic nitrogen regulator. We demonstrate that uridylylated PII (but not underivatized PII) activates NagB more then 10 fold at low concentrations of substrate, while NanE increases NagB activity over 2 fold. Activation of NagB by HPr and uridylylated PII as well as by NanE and HPr (but not NanE and U-PII) is synergistic. Specific functions for the regulation of NagB by its three protein activators are proposed. Each regulatory agent is suggested to mediate signal transduction in response to a different stimulus.

**Importance**

The regulation of amino sugar utilization is important for survival and pathogenicity in a competitive environment such as the human gastrointestinal tract. For example, strains of *V. cholerae* that lack the neuraminic acid utilization lyase, NanA, are defective in intestinal colonization in a mouse infection model, and sialic acid has been shown to be a major virulence determinant in the pathogenesis of Haemophilus influenzae.

NagB, glucosamine 6-phosphate deaminase, is essential for amino sugar utilization and is known to be allosterically regulated by N-acetyl-glucosamine 6-phosphate (GlcNAc-6P) and the phosphocarrier protein of the bacterial phosphotransferase system, HPr, in *Escherichia coli*. We provide evidence that NanE, GlcNAc-6P epimerase, and the uridylylated PII protein also allosterically activate NagB by direct protein-protein interactions. NanE is essential for neuraminic acid (NANA) and N-acetylmannosamine (ManNAc) utilization, and the PII protein is known to be a central metabolic nitrogen regulator.

**Keywords:** glucosamine 6-phosphate deaminase/isomerase, NagB; allosteric regulation; protein-protein interactions; nitrogen regulator, PII**;** N-acetylglucosamine 6-phosphate epimerase, NanE; signal transduction.

**Introduction**

N-acetylglucosamine (GlcNAc) is present in glycans in human milk (1) and in animal cell surface mucus (2). The human gastrointestinal tract is heavily colonized by bacteria with most species belonging to the phyla of Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria (3). The microbiome representative, *E. coli*, tightly controls the utilization of amino sugars (4), stable sources of carbon and ammonia in highly competitive neighbourhoods.

GlcNAc is a constituent of chitin, other polysaccharides, glycolipids and glycoproteins, and N-acetylmannosamine (ManNAc) and mannosamine (ManN), are metabolites of neuraminic acid (NANA; sialic acid) utilization. NANA is essential for the synthesis of some polysaccharides and the glycosylation of certain proteins and lipids in eukaryotes and prokaryotes. Exogenous NANA is utilized by *E. coli* via the transporter, NanT, and further hydrolyzed by a lyase, NanA, to produce pyruvate and ManNAc. NANA utilization is essential for the colonization of pathogens. For example *Vibrio cholerae* and Haemophilus influenzae require sialic acid for virulence (5,6)*.* ManNAc and ManN are taken up from the growth medium by the ManXYZ enzyme complex of the bacterial phosphoenolpyruvate:sugar phosphotransferase system (PTS) and concomitantly phosphorylated to ManNAc-6P and Man-6P, respectively (7) (Fig. 1). In contrast, ManNAc, produced from NANA hydrolysis, is phosphorylated in the cell by an ATP-dependent ManNAc kinase – NanK.

The pathway for N-acetylmannosamine utilization includes an epimerase, NanE, which converts ManNAc-6P to GlcNAc-6P as part of the NANA and ManNAc utilization pathways (Fig. 1). The NanR transcriptional regulator controls expression of the operon, *nanA-nanT-nanE-nanK* for NANA utilization, and NANA is the inducer that causes NanR to dissociate from its high affinity binding site in the *nanATEK* operon (8)*.* NagA further deacetylates GlcNAc-6P to glucosamine 6-phosphate (GlcN-6P). GlcNAc-6P is available from the catabolism of ManNAc and NANA as well as from extracellular GlcNAc, transported into the cell by the bacterial PTS (9,10). Glucosamine isomerase/deaminase, NagB, provides the last step in the catabolic pathway, converting GlcN-6P to NH3 and fructose 6-phosphate (Fru-6P), an intermediate of glycolysis. This enzyme is essential for the utilization of amino sugars in *E. coli* and is known to be allosterically activated by an intermediate of the GlcNAc metabolic pathway, GlcNAc-6P (11). Orthologs of this enzyme are present not only in bacteria, but also in mammals and other organisms.

The biosynthetic pathway producing UDP-GlcNAc for incorporation into cell wall components involves the glmS, glmM and glmU gene products and utilizes the cytoplasmic GlcN-6P pool. NagB redirects GlcN-6P to the glycolytic pathway and is thus important and tightly regulated by two previously recognized factors, GlcNAc-6P as noted above, and a primary constituent of the PTS, HPr, a sensor of extracellular PTS sugar substrates (12) including GlcNAc, ManNAc and GlcN.

NagB interactome data (Babu et al., manuscript submitted), reproduced in Table 1, suggest that NagB interacts with several cellular proteins, including the nitrogen-related signal transduction PII protein, NanE, proline aminopeptidase, PepP, a nitroreductase (capable of reducing nitrofurazone and quinones), NfsB, and even the riboflavin biosynthetic enzymes, RibA and RibB. The work reported here shows that NagB is activated by NanE in the presence or absence of GlcNAc-6P and by the PII protein covalently modified by uridylylation, an indicator of nitrogen availability.

The uridylylated PII protein (U-PII), is generated by the glutamine/-ketoglutarate ratio-sensing uridylyltransferase/urindylyl-removing enzyme (GlnD) (13). Adenylylation of glutamine synthetase GlnA is stimulated by the PII protein (GlnB), and deadenylylation is stimulated by U-PII, thus comprising a dual bicyclic cascade. We show that activation of NagB by U-PII in the presence of low concentrations of GlcNAc-6P leads to an increase in activity of more then 10 fold. Synergistic effects of NagB activation for HPr/U-PII and HPr/NanE have been detected. These observations are rationalized.

**Results**

**Kinetic measurements of NagB activity.**We examined the effects of His-tagged, purified, recombinant proteins, NanE, PII (GlnB), GlnD, Zwf, Tsf, and BglB on NagB activity. Activity of NagB was measured after purification with a His-tag and followed by proteolytic removal of the His-tag resulting in the Ser-1 NagB derivative (see Materials and Methods). Met-1 in NagB has been shown to play a role in activation by GlcNAc-6P (14), possibly explaining the higher Ka of 2.1 mM (Fig S1) in the activation of NagB by GlcNAc-6P reported here as compared with that reported previously (11). *E. coli* NagB activity was measured by following the increase in absorbance at 340 nm resulting from the reduction of NADP in a coupled assay involving Pgi and Zwf (see Materials and Methods). This assay is based on the conversion of GlcN-6P to fructose 6-phosphate by NagB, followed by isomerization to glucose 6-phosphate by Pgi and further oxidation of glucose 6-phosphate to gluconate 6-phosphate by Zwf. We showed that Zwf at the conditions used has no effect on NagB activity (data not shown).

**The effects of PII and NanE on NagB activity measured at a fixed concentration of both GlcNAc-6P and GlcN-6P.**Allosteric regulation by GlcNAc-6P is known for *E. coli* NagB and the non-orthologous deaminase/isomerase, NagB-II, from *Shewanella*, which belongs to the sugar isomerase protein family (15). To demonstrate regulation of *E. coli* NagB, suggested by the protein-protein interactome data (Table 1), we examined the effects of purified NanE (0.7 M), PII (GlnB) (1 M), -phospho-glucosidase (BglB) (0.2 M) and the translation elongation factor (Tsf) (2 M). The latter two proteins were used as negative controls for the activation of NagB. Based on activity measurements for NagB, with 0.4 mM of the allosteric effector, GlcNAc-6P, and 4 mM of the substrate, GlcN-6P, NanE and GlnB both activated NagB, as shown in Fig. 2. There was no effect when either purified Tsf or purified BglB was added (Fig. 2). The activation effect of NanE on NagB activity was ≈2 fold. However, a much greater effect was observed with purified uridylylated PII. In this experiment, the increase of NagB activity in the presence of freshly purified PII was 7-fold compared to the negative controls when no protein, Tsf or BglB was added.

**PII (GlnB)-dependent activation of NagB depends on the uridylylation state of GlnB.**The kinetics for NagB in the presence of PII at different stages of PII modification involving uridylylation by GlnD were measured (Fig. 3A). The reaction mixture for the covalent modification of PII included: 0.2 M Tris, pH 8, 0.3 mM ATP, 3 mM UTP, 1 mM DTT, 200 nM GlnD, 50 mM KCl, 1 mM -ketoglutarate (-KG) and 25 mM MgSO4. After incubating this reaction mixture for 20 min, the partially uridylylated PII fwas collected, and after 4 hours at 25 oC, GlnD generated fully uridylylated PII (U-PII). For both PII forms, the kinetics for NagB activation were measured using 0.4 mM GlcNAc-6P. The assay mixture included 0.5 mM ATP and 1 mM UTP in addition to the NagB assay mixture. The kinetics revealed increased activity in the presence of fully uridylylated 0.4 M U-PII (Fig. 3B) at pH 8.

The effect was greater at pH 7.5 than at pH 8, due to the allosteric behaviour of NagB, resulting from an increased Hill coefficient (16). Accordingly, U-PII at 0.2 M substantially decreased the NagB Khalf for GlcN-6P, when the kinetics were measured at pH 7.5. The increase in NagB activity was more than 10-fold in the presence of U-PII at low concentrations of the substrate, GlcN-6P.

**NanE-dependent activation of NagB is not dependent on GlcNAc-6P.**NanE activation of NagB was measured in the presence and absence of GlcNAc-6P. The activity measurements with respect to GlcN-6P concentration are shown in Fig. 4. The effect of 0.7 M NanE at pH 7.8 is shown in Fig. 4A without the effector, GlcNAc-6P. Under these conditions, NagB should be largely in the poorly active T-state. Increased activity of more then 2-fold was observed, particularly at low concentrations of the substrate, GlcN-6P. The kinetics with 0.2 mM GlcNAc-6P (the partially activated state of NagB), measured at pH 6.8 in the presence and absence of 0.7 M NanE, are presented in Fig 4B. A somewhat larger increase in activity due to NanE was observed at concentrations of GlcN-6P of more then 2 mM, although the activation appeared to be less at low GlcN-6P concentrations (data not shown).

**Synergistic effects of HPr- and U-PII-dependent and of HPr- and NanE-dependent activation of NagB measured at fixed concentrations of both GlcNAc-6P and GlcN-6P.** HPr has previously been shown to activate NagB I. Rodionova, in press, 2017). The synergistic effects of HPr and U-PII at non-saturating concentrations on NagB activity, were measured with 0.4 mM of the allosteric effector, GlcNAc-6P, and 4 mM substrate, GlcN-6P (Fig. 2B). The 0.1 ml of assay mixture contained 0.2 M Tris, pH 7.5, 1 mM ATP, 1 mM DTT, 2 mM phosphate, 20 mM KCl, 10 mM MgSO4,2 mM NADP, 1.2 U Pgi and 1.2 U Zwf. The activation effect for U-PII was 2-fold and for HPr was 4 fold. The cumulative synergistic effect when both U-PII and HPr were added together was 10-fold when the same concentrations of those proteins were present (0.05 M and 0.5 M respectively).

When NanE and HPr were added together in the same assay mixture, but with 0.3 mM of the allosteric effector, GlcNAc-6P, and 3 mM substrate, GlcN-6P (Fig. 5), there was substantial synergism at concentrations of HPr higher then 0.8 M (Fig. 5). Titration with non-phosphorylated HPr showed sigmoidal kinetics in the presence of 0.3M NanE.

**Discussion**

The PII protein is known to be a regulator of both the activity and the synthesis of glutamine synthetase (GS, GlnA) in enteric bacteria and of nitrogen metabolism in many other bacteria, archaea and eukaryotes in response to the availability of a nitrogen source (Fig. 1) (17-21). The pathways that regulate *glnA* gene expression and GS enzymatic activity both involve the covalent modification of proteins (Fig. 1). The regulation of GS activity involves de-adenylylation for activation and adenylylation for inactivation with both reactions catalyzed by the same enzyme, adenylyltransferase/adenylylase, GlnE. The direction of GS derivatization is dictated by the PII protein, the state of which is also regulated by reversible covalent modification by uridylylation catalyzed by GlnD, another bifunctional enzyme regulated oppositely by KG and glutamine. The modified form, U-PII, is essential for the de-adenylylation reaction acting on GS.

We found that the modified form of PII, U-PII, activates NagB. Coordinate activation of both NagB and GS by U-PII makes teleological sense since activation of the former releases NH3, while activation of GS facilitates its incorporation into glutamine for the synthesis of numerous other nitrogenous compounds. The effects and consequences of the NagB allosteric interactions can be summarized as follows: 1. The presence of amino sugars (or any PTS sugar substrate) in the medium de-phosphorylates HPr and activates NagB. 2. Increase in the cytoplasmic GlcNAc-6P concentrations promotes high levels of *nagB* expression and high activity of NagB. 3. The activation of NagB by U-PII, promotes successful utilization of amino sugars, thereby increasing levels of both carbon and nitrogen in the cell. 4. GS will be converted to the unmodified active form, allowing the incorporation of the NH3, released from GlcN-6P, into glutamine. 5. NanE activation of NagB only occurs when NANA is available, promoting high level expression of the *nanE* gene.

The pathways for the utilization of different amino sugars, NANA, ManNAc-6P and GlcNAc-6P, converge with the production of GlcN-6P, the substrate of NagB (Fig. 1). If GlcN or GlcNAc is transported into the cell by the PTS, HPr, present in the non-phosphorylated form, will activate NagB in response to the availability of its sugar substrates (I. Rodionova et al., 2017). But what if NANA is utilized? During exogenous NANA utilization, the transporter is NanT, and no accumulation of GlcNAc occurs; this means that the PTS protein, HPr, should be largely phosphorylated (HPr-P), and HPr-P has no effect on the activity of NagB (I. Rodionova et al., 2017). Under these conditions, *nanE* gene expression is induced in response to the availability of cytoplasmic NANA, so that even in the absence of GlcNAc-6P, NagB will be activated by NanE. Thus, we propose that NanE activates NagB when cytoplasmic or exogenously derived NANA is available and metabolized. HPr activates NagB only when the PTS is used for sugar uptake, and U-PII activates NagB primarily under nitrogen limiting conditions. Thus, NanE transmits a signal indicating the presence of cytoplasmic NANA, HPr signals the availability of an extracellular PTS sugar substrate, and U-PII signals nitrogen deficiency since GlnD, which uridylylate PII, senses the ratio of cytoplasmic glutamine to KG. These signal transducing systems allow the bacteria to respond to at least three different signals, all converging to regulate the activity of glucosamine-6-phosphate deaminase. We have further shown that HPr and U-PII as well as HPr and NanE act synergistically under appropriate conditions, greatly enhancing the activating effect of either one. In contrast, U-PII and NanE had no synergistic effect, possibly suggesting that they bind to the same site or overlapping sites of NagB or the NagB-HPr complex.

**Materials and methods**

## GlcNAc-6P, GlcN-6P, NADP, UTP, L-glutamine, -ketoglutarate, dithiothreitol (DTT) and other chemicals were purchased from Sigma-Aldrich, USA.

**Cloning nagB into pMST3.**The *nagB* gene, encoding GlcN-6P deaminase, NagB, was PCR amplified from the *E. coli* BW25113 chromosome using oligos nagB-Bam-F: ataggatccagactgatccccctgactaccgctgaac and nagB-Sal-R: ctcgtcgacttacagacctttgatattttctgcttc. The product was gel purified, digested with *BamH*I and *Sal*I, and then cloned into the pSMT3 vector digested with the same restriction endonucleases. Individual clones were confirmed by colony PCR and subsequently by DNA sequencing. The resultant recombinant plasmid, pMST3-*nagB*, carried the *nagB* structural gene (without the first codon) fused to the 3’ end of the SUMO gene (without its stop codon) encoding the SMT3-His-tag. Expression of “SUMO:*nagB*” was under the control of the T7 promoter. The SMT3 tag, present in the fusion protein, was removed by the Ulp1 Sumo protease. The resultant NagB enzyme (Ser1-NagB) has a serine residue instead of the first methionine residue (Met1-NagB).

**Protein purification.**Recombinant proteins, NagB, NanE, PII (GlnB), GlnD, Zwf, Pgi, Tsf and BglB, all containing an N-terminal His6 tag, were overexpressed in E. coli and purified using Ni2+-chelating chromatography. The E. coli OE strains for NanE, PII (GlnB), GlnD, Zwf, Pgi, Tsf and BglB, from the ASKA collection (22), were used for protein purification. Strains were grown in LB medium (50 ml), induced by addition of 0.6 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and harvested after 4 h of shaking. Rapid purification of recombinant proteins on Ni-nitrilotriacetic acid (NTA) agarose minicolumns was performed as described previously (23). The cells were harvested and re-suspended in 20 mM HEPES buffer, pH 7, containing 100 mM NaCl, 2 mM β-mercaptoethanol, and 0.3% Brij 35 with 2 mM phenylmethylsulfonyl fluoride. Cells were lysed by incubation with lysozyme (1 mg/ml) for 30 min, followed by a freeze-thaw cycle and sonication. After centrifugation, the supernatant was loaded onto a Ni-NTA agarose minicolumn (0.3 ml) from Qiagen Inc. (Valencia, CA). After bound proteins were washed with 2 ml of At-buffer containing 50 mM Tris-HCl buffer (pH 8), 0.5 M NaCl, 5 mM imidazole and 0.3 % Brij 35, they were eluted with 0.3 ml of the same buffer supplemented with 250 mM imidazole. Protein size, expression level and purity were monitored by SDS-PAGE. All proteins were obtained in high yield (≈ 1 mg) and purity (80 to 90%). Protein concentrations were measured using the Bradford assay kit (Biorad).

For the purified NagB the Sumo-tag was proteolytically removed using the Ulp1 Sumo protease after adding 1M urea to the buffer used for protein purification (24) at 4 oC. Then the buffer was changed to At-buffer by dialysis, and NagB was collected from a Ni-NTA agarose column in the flow-through fractions.

**NagB activity measurements***.* Activity of the purified recombinant NagB protein was routinely assayed in a cuvette at 37°C using the standard enzymatic coupling assay involving phosphoglucose isomerase (Pgi) and glucose 6-phosphate dehydrogenase (Zwf) by measuring the increase in absorbance at 340 nm resulting from the reduction of NADP as described previously (15). NagB kinetics as a function of the GlcN-6P concentration were measured using 0 - 0.4 mM GlcNAc-6P in the presence of 0.2 M Tris, pH 6.5 - 8.1, 5 mM phosphate, 10 mM MgSO4, 3 mM NADP, 50 mM KCl, 0.9 U Zwf and 0.9 U Pgi.

**GlnD-dependent uridylylation of the PII protein.**GlnDwas assayedin a 1 ml assay mixture containing the purified PII recombinant protein at a concentration of 10 M. The reaction for the covalent modification of PII included: 0.2 M Tris, pH 7.5, 1 mM ATP, 3 mM UTP, 1 mM DTT, 200 nM GlnD, 50 mM KCl and 0.5 mM -ketoglutarate. The reaction mixture was incubated at 30oC for 1 hour.

***Acknowlegments***

We thank Jimmy Do for appreciated help with plasmid purification and Dr. Zhongge Zhang for constructing of the *nagB* overexpression strain. **Funding:** this work was supported by NIH grant GM109895. **Author contributions:** I.R. completed the experiments, M.S. supervised the study, I.R. and M.S. analyzed the data and wrote the manuscript.

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