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

Irina A. Rodionova [1,#], Norman Goodacre[2], Mohan Babu[3], Andrew Emili[4], Peter Uetz[2], and Milton H. Saier, Jr.[1,*]

Department of Molecular Biology, Division of Biological Sciences, University of California at San Diego, La Jolla, CA 92093-0116, USA[1]; Center for the Study of Biological Complexity, Virginia Commonwealth University, Richmond, VA 23284[2]; Department of Biochemistry, Research and Innovation Centre, University of Regina, Regina, Canada[3]; Donnelly Centre, University of Toronto, Toronto, Ontario, Canada[4]

Running Head: Regulation of NagB glucosamine 6-phosphate deaminase (NagB) by protein-protein Interactions

#Address correspondence to Irina A. Rodionova, irodionova@ucsd.edu

*Corresponding author:

Telephone: (858) 534-4084

Fax: (858) 534-7108

Email: msaier@ucsd.edu

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 by NanE and U-PII) is synergistic, and the modelling suggests specific residues potentially involved in complex formation. 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. The 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 allosterically regulated by N-acetyl-glucosamine 6-phosphate (GlcNAc-6P). 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 ManN-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 6-phosphate isomerase/deaminase, NagB, provides the last step in the catabolic pathway, converting GlcN-6P to NH_3 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 derivatization involving the glutamine/α-ketoglutarate ratio-sensing uridylyltransferase/uridylyl-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 HPr/U-PII and HPr/NanE on NagB activation have been detected. The modelling of HPr/U-PII/NagB and HPr/NanE/NagB complexes formation confirmed the possibility that the two proteins (HPr/U-PII or HPr/NanE) can simultaneous interact with NagB, although U-PII/ NanE cannot. 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 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 K_a 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, under the conditions used, has no effect on NagB activity (data not shown).

The effects of PII and NanE on NagB activity measured at fixed concentrations 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 μM), PII (GlnB) (1 μM), β-phospho-glucosidase (BglB) (0.2 μM) and the translation elongation factor (Tsf) (2 μM). 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 α-ketoglutarate (α-KG) and 25 mM $MgSO_4$. After incubating this reaction mixture for 20 min, the partially uridylylated PII was collected, and after 4 hours at 25 ^{o}C, 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 usual NagB assay mixture. The kinetics revealed increased activity in the presence of fully uridylylated 0.4 μM 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 μM substantially decreased the NagB K_{half} 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 μM 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 μM 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 et al., 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 $MgSO_4$, 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 μM and 0.5 μM, 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 μM (Fig. 5). Titration with non-phosphorylated HPr showed sigmoidal kinetics in the presence of 0.3μM NanE.

Modeling of HPr/U-PII and of HPr/NanE binding to NagB. There is structural evidence that U-PII (5L9N:A) and activated HPr (3CCD:A), as well as NanE (SMR model, NanE_Ecoli:A) and HPr, exert their synergistic effects on NagB through simultaneous binding. From HADDOCK protein-protein docking, it was possible to find energetically favorable conformations for the binary complexes of HPr/NagB, U-PII/NagB, and NanE/NagB where HPr would not sterically occlude either U-PII or NanE, but U-PII and NanE would sterically exclude each other. This was revealed by aligning the three separate binary complexes by NagB and observing the positions of HPr, U-PII, and NanE. All three binary complexes had significant HADDOCK and z-scores, and were in the top 4 clusters of docked results. All three binary complexes also were either the largest or second-largest cluster in their respective docking outputs.

One noteworthy aspect of the NagB interface (collective, adjacent binding sites for U-PII, NanE, and HPr) is that it is highly disordered. There is almost no secondary structure at all although there are loops. Also noteworthy is that this disordered area is large, approximately one-fourth of the entire surface area of NagB. Possibly, as a result of the disordered binding surface across the 4 HPr/NagB docking clusters

with significant scores, HPr exhibits considerable hinging but keeps nearly the same contacts with NagB. This suggests that the NagB interface is highly flexible. Analysis of the models suggests that HPr, U-PII, and NanE bind very close to each other on NagB. It is possible that the flexibility of the NagB interface allows for the simultaneous binding of HPr/U-PII, as well as HPr/NanE. However, an entire alpha helix from NanE overlaps with the core of the U-PII binding site, making simultaneous binding of these two NagB activators unlikely, no matter what extent of flexibility is allowed. Thus, NanE cannot be hinged away from U-PII, as HPr can. Therefore, the shown modelling of the HPr/U-PII/NagB and HPr/NanE/NagB complexes revealed the residues involved in the simultaneous interaction of the two proteins (HPr/U-PII or HPr/NanE) with NagB (Table S1), and excluded the possibility for U-PII/ NanE simultaneous binding to NagB.

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 αKG 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 NH_3, 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 NH_3 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 PTS 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

277 phosphorylated (HPr-P), and HPr-P has no effect on the activity of NagB (I.
278 Rodionova et al., 2017, in press). Under these conditions, *nanE* gene expression is
279 induced in response to the availability of cytoplasmic NANA, so that even in the
280 absence of GlcNAc-6P, NagB will be activated by NanE. Thus, we propose that NanE
281 activates NagB when cytoplasmic or exogenously derived NANA is available and
282 metabolized. HPr activates NagB only when the PTS is used for sugar uptake, and U-
283 PII activates NagB primarily under nitrogen limiting conditions. Thus, NanE
284 transmits a signal indicating the presence of cytoplasmic NANA, HPr signals the
285 availability of an extracellular PTS sugar substrate, and U-PII signals nitrogen
286 deficiency since GlnD, which uridylylates PII, senses the ratio of cytoplasmic
287 glutamine to αKG. These signal transducing systems allow the bacteria to respond to
288 at least three different signals, all converging to regulate the activity of glucosamine-
289 6-phosphate deaminase. We have further shown that HPr and U-PII as well as HPr
290 and NanE act synergistically under appropriate conditions, greatly enhancing the
291 activating effect of either one. In contrast, U-PII and NanE had no synergistic effect,
292 possibly suggesting that they bind to the same site or overlapping sites of NagB or
293 the NagB-HPr complex.
294
295 **Materials and methods**
296 GlcNAc-6P, GlcN-6P, NADP, UTP, L-glutamine, α-ketoglutarate, dithiothreitol (DTT)
297 and other chemicals were purchased from Sigma-Aldrich, USA.
298
299 **Cloning nagB into pMST3.** The *nagB* gene, encoding GlcN-6P deaminase, NagB, was
300 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 His_6 tag, were overexpressed in *E. coli* and purified using Ni^{2+}-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 ^{o}C. 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 $MgSO_4$, 3 mM NADP, 50 mM KCl, 0.9 U Zwf and 0.9 U Pgi.

GlnD-dependent uridylylation of the PII protein. GlnD was assayed in a 1 ml assay mixture containing the purified PII recombinant protein at a concentration of 10 μM. 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 α-ketoglutarate. The reaction mixture was incubated at 30°C for 1 hour.

Structural modeling of the U-PII/ HPr and NanE/HPr proteins interaction with NagB HPr, NanE, and U-PII were each docked to NagB individually using the HADDOCK webserver with CPORT predicted interface residues as active and passive restraints. All structures from clusters with negative z-scores (below average energy scores, among clusters of the top 200 structures) were considered in the modeling. Docked complexes for HPr/NagB, NanE/NagB, and U-PII/NagB were aligned by NagB in Pymol. The #4, #1, and #3 clusters for HPr/NagB, NanE/NagB, and U-PII/NagB were selected for further analysis, as these allowed unobstructed orientations for HPr and NanE, as well as HPr and U-PII, in their bound state with NagB. The HPr/NagB and NanE/NagB clusters were both the largest (greatest number of docking models) clusters from their respective docking runs, while the U-PII/NagB cluster was the second-largest. The PDB IDs and chains used for NagB, HPr, and U-PII were 1FS5:A, 3CCD:A, 5L9N:A. 1FS5:A is a structure of the open, "R" conformation of NagB, and 5L9N:A is a structure of uridylylated PII. For NanE, the full-length Swiss Model Repository model based on the template 3IGS:A (79.7% sequence identity) was used.

Acknowledgments

We thank Jimmy Do for appreciated help with plasmid purification and Dr. Zhongge Zhang for constructing 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 P.U. and N. J. performed modeling for protein-protein interaction, MB conducted the interactome studies reported in Table 1.

Conflicts of Interest

The authors declare no conflicts of interest.

References

1. **Garrido D, Ruiz-Moyano S, Mills DA.** 2012. Release and utilization of N-acetyl-D-glucosamine from human milk oligosaccharides by Bifidobacterium longum subsp. infantis. Anaerobe **18:**430-435.
2. **Koropatkin NM, Cameron EA, Martens EC.** 2012. How glycan metabolism shapes the human gut microbiota. Nat Rev Microbiol **10:**323-335.
3. **Tailford LE, Crost EH, Kavanaugh D, Juge N.** 2015. Mucin glycan foraging in the human gut microbiome. Front Genet **6:**81.
4. **Vimr ER, Troy FA.** 1985. Identification of an inducible catabolic system for sialic acids (nan) in Escherichia coli. J Bacteriol **164:**845-853.

5. **Jenkins GA, Figueira M, Kumar GA, Sweetman WA, Makepeace K, Pelton SI, Moxon R, Hood DW.** 2010. Sialic acid mediated transcriptional modulation of a highly conserved sialometabolism gene cluster in Haemophilus influenzae and its effect on virulence. BMC Microbiol **10:**48.

6. **Almagro-Moreno S, Boyd EF.** 2009. Sialic acid catabolism confers a competitive advantage to pathogenic vibrio cholerae in the mouse intestine. Infect Immun **77:**3807-3816.

7. **Plumbridge J, Vimr E.** 1999. Convergent pathways for utilization of the amino sugars N-acetylglucosamine, N-acetylmannosamine, and N-acetylneuraminic acid by Escherichia coli. J Bacteriol **181:**47-54.

8. **Walters DM, Stirewalt VL, Melville SB.** 1999. Cloning, sequence, and transcriptional regulation of the operon encoding a putative N-acetylmannosamine-6-phosphate epimerase (nanE) and sialic acid lyase (nanA) in Clostridium perfringens. J Bacteriol **181:**4526-4532.

9. **Barabote RD, Saier MH, Jr.** 2005. Comparative genomic analyses of the bacterial phosphotransferase system. Microbiol Mol Biol Rev **69:**608-634.

10. **Deutscher J, Ake FM, Derkaoui M, Zebre AC, Cao TN, Bouraoui H, Kentache T, Mokhtari A, Milohanic E, Joyet P.** 2014. The bacterial phosphoenolpyruvate:carbohydrate phosphotransferase system: regulation by protein phosphorylation and phosphorylation-dependent protein-protein interactions. Microbiol Mol Biol Rev **78:**231-256.

11. **Alvarez-Anorve LI, Bustos-Jaimes I, Calcagno ML, Plumbridge J.** 2009. Allosteric regulation of glucosamine-6-phosphate deaminase (NagB) and growth of Escherichia coli on glucosamine. J Bacteriol **191:**6401-6407.

12. **Gabor E, Gohler AK, Kosfeld A, Staab A, Kremling A, Jahreis K.** 2011. The phosphoenolpyruvate-dependent glucose-phosphotransferase system from Escherichia coli K-12 as the center of a network regulating carbohydrate flux in the cell. Eur J Cell Biol **90:**711-720.

13. **van Heeswijk WC, Molenaar D, Hoving S, Westerhoff HV.** 2009. The pivotal regulator GlnB of Escherichia coli is engaged in subtle and context-dependent control. FEBS J **276:**3324-3340.

14. **Lara-Gonzalez S, Dixon HB, Mendoza-Hernandez G, Altamirano MM, Calcagno ML.** 2000. On the role of the N-terminal group in the allosteric function of glucosamine-6-phosphate deaminase from Escherichia coli. J Mol Biol **301:**219-227.

15. **Yang C, Rodionov DA, Li X, Laikova ON, Gelfand MS, Zagnitko OP, Romine MF, Obraztsova AY, Nealson KH, Osterman AL.** 2006. Comparative genomics and experimental characterization of N-acetylglucosamine utilization pathway of Shewanella oneidensis. J Biol Chem **281:**29872-29885.

16. **Montero-Moran GM, Lara-Gonzalez S, Alvarez-Anorve LI, Plumbridge JA, Calcagno ML.** 2001. On the multiple functional roles of the active site histidine in catalysis and allosteric regulation of Escherichia coli glucosamine 6-phosphate deaminase. Biochemistry **40:**10187-10196.

17. **Lillo C.** 2008. Signalling cascades integrating light-enhanced nitrate metabolism. Biochem J **415:**11-19.

18. **Muro-Pastor MI, Reyes JC, Florencio FJ.** 2005. Ammonium assimilation in cyanobacteria. Photosynth Res **83:**135-150.

19. **Francis SH, Engleman EG.** 1978. Cascade control of E. coli glutamine synthetase. I. Studies on the uridylyl transferase and uridylyl removing enzyme(s) from E. coli. Arch Biochem Biophys **191:**590-601.

20. **Son HS, Rhee SG.** 1987. Cascade control of Escherichia coli glutamine synthetase. Purification and properties of PII protein and nucleotide sequence of its structural gene. J Biol Chem **262:**8690-8695.

21. **Arcondeguy T, Jack R, Merrick M.** 2001. P(II) signal transduction proteins, pivotal players in microbial nitrogen control. Microbiol Mol Biol Rev **65:**80-105.

22. **Kitagawa M, Ara T, Arifuzzaman M, Ioka-Nakamichi T, Inamoto E, Toyonaga H, Mori H.** 2005. Complete set of ORF clones of Escherichia coli ASKA library (a complete set of E. coli K-12 ORF archive): unique resources for biological research. DNA Res **12:**291-299.

23. **Rodionova IA, Li X, Thiel V, Stolyar S, Stanton K, Fredrickson JK, Bryant DA, Osterman AL, Best AA, Rodionov DA.** 2013. Comparative genomics and functional analysis of rhamnose catabolic pathways and regulons in bacteria. Front Microbiol **4:**407.

24. **Hay RT.** 2007. SUMO-specific proteases: a twist in the tail. Trends Cell Biol **17:**370-376.

457 **Table 1.**
458

	Protein location	Score	Protein name and EC number
DdpA	PE	9.9	D,D-dipeptide-binding periplasmic protein DdpA
GcvT	MA	6.1	Aminomethyltransferase (glycine cleavage system T protein) (EC 2.1.2.10)
NfsB	MA	6.1	Nitroreductase
PepP	CY	5.9	Xaa-Pro aminopeptidase
GlnH	PE	5.9	Glutamine ABC transporter, substrate-binding protein GlnH
GcvP	MA	5.8	Glycine dehydrogenase [decarboxylating] (glycine cleavage system P protein) (EC 1.4.4.2)
YifE	MA	5.7	UPF0438 protein YifE
CysS	CY	5.7	Cysteinyl-tRNA synthetase (EC 6.1.1.16)
HemB	CY	5.6	Porphobilinogen synthase (EC 4.2.1.24)
GpmM	CY	5.5	2,3-bisphosphoglycerate-independent phosphoglycerate mutase (EC 5.4.2.1)
BglA	CY	5.4	6-phospho-beta-glucosidase (EC 3.2.1.86)
NanE	CY	5.4	N-acetylmannosamine-6-phosphate 2-epimerase (EC 5.1.3.9)
RibC	CY	5.4	Riboflavin synthase alpha chain RibC
Zwf	CY	5.4	Glucose-6-phosphate 1-dehydrogenase (EC 1.1.1.49)
RibB	MA	5.4	3,4-dihydroxy-2-butanone 4-phosphate synthase (EC 4.1.99.12)
GlnB	CY	5.3	Nitrogen regulatory protein P-II
ProC	CY	5.3	Pyrroline-5-carboxylate reductase (EC 1.5.1.2)
PpiA	PE	5.3	Peptidyl-prolyl cis-trans isomerase ppiA precursor (EC 5.2.1.8)
YbbN	CY	5.3	Thioredoxin domain-containing protein EC-YbbN
PtsH	MA	5.3	Phosphocarrier protein
FumA	MA	5.3	Fumarate hydratase class I, aerobic (EC 4.2.1.2)
BioB	CY	5.3	Biotin synthase (EC 2.8.1.6)
NemA	CY	5.3	Flavoprotein NADH-dependent oxidoreductase
RibA	CY	5.3	GTP cyclohydrolase II

459 PE, periplasm; MA, membrane; CY, cytoplasm
460

461

462

Table 2.

	NagB, pH 8.1 (GlcNAc-6P, 0.4mM)			NagB, pH 7.8 (no GlcNAc-6P)		NagB, pH 6.8 (GlcNAc-6P, 0.2mM)	
NagB activity	+U-PII	-PII	+PII	+NanE	-NanE	+NanE	-NanE
Vmax, U/mg	90±11	11.3±1.8	16.8±2.0	5.4±0.4	3.5±0.9	28.9±3.5	13±1.3
h	1.3±0.3	1.6±0.8	1.0±0.4	2.1±0.3	2.1±0.8	1.9±0.6	1.4±0.4
K_{half}, mM	3.6±0.8	2.9±0.7	2.3±0.6	4.8±0.5	8.0±2.0	2.0±0.4	1.9±0.4

FOOTNOTES

This work was supported by NIH grant GM109895.

Abbreviations used are: NagB, Glucosamine 6-phosphate isomerase/deaminase; HPr, histidine-phosphorylatable phosphocarrier protein; PTS, phosphotransferase system; PII, nitrogen regulator**;** NanE, N-acetylglucosamine 6-phosphate epimerase;

Figure legends

FIG. 1. Amino sugar utilization in *E. coli*. Protein-protein interactions of HPr-NagB, U-PII-NagB and NanE-NagB activate NagB by increasing the affinity of the enzyme for its substrate, GlcN-6P and/or increasing the V_{max}. NagB, glucosamine 6-phosphate deaminase. These proteins are indicated by the white rectangles adjacent to the upward pointing arrows. NagA, N-acetyl-glucosamine 6-phosphate deacetylase; NANA, N-acetyl-D-neuraminic acid; NanA, NANA aldolase/lyase; PTS,

482 phosphotransferase system; ManXYZ, the mannose PTS system; NanT, the NANA
483 transporter; NanK, ManNAc kinase; GS, glutamine synthetase; PII (GlnB), nitrogen
484 regulator; GlnD, uridylyltransferase/uridylylase for the PII protein, GlnE;
485 adenylyltransferase/adenylase
486
487 **FIG. 2** Protein-protein interaction-dependent activation of NagB in the presence of a
488 non-saturating concentration of the allosteric effector, GlcNAc-6P (0.4 mM). A.
489 Activity was measured at 5 mM GlcN-6P in the presence of NanE (0.7 mM), PII
490 (GlnB) (1 mM) and /or HPr 0.5 mM. BglB (0.2 mM) and Tsf (2 mM) were included as
491 negative controls. B. Activity was measured at 2 mM GlcN-6P in the presence of HPr
492 (0.5 mM), U-PII (0.05 mM) or a combination of the same concentrations of HPr and
493 U-PII, at pH 7.5.
494
495 **FIG. 3** Allosteric activation of NagB by the uridylylated PII protein (U-PII). A. The
496 kinetics were measured as a function of the GlcN-6P concentration (0 to 10 mM) in
497 the presence of the allosteric effector, 0.4 mM GlcNAc-6P in the absence (diamonds)
498 or the presence (squares) of 0.4 mM partially uridylylated U-PII (squares), 0.4 mM
499 fully uridylylated U-PII (filled circles) or 0.6 mM free PII (open circles), all at pH 8.
500 The assay is described in Materials and Methods. B. Steady-state kinetics of NagB
501 measured as a function of the GlcN-6P concentration (0 to 14 mM) in the presence of
502 the allosteric effector, 0.2 mM GlcNAc-6P in the absence (triangles) or presence
503 (filled circles) of 0.2 mM U-PII, at pH 7.5. One unit of activity = 1 mMol of product
504 formed/mg protein/min

505

506 **FIG. 4** Allosteric activation of NagB by NanE. A. Steady-state kinetics of NagB were
507 determined as a function of GlcN-6P concentration (0 to 15 mM) in the absence
508 (squares) and presence (circles) of 0.7 mM NanE at pH 7.8. B. The same steady-state
509 kinetics of NagB activity at pH 6.8 and in the presence of the allosteric effector,
510 0.2mM GlcNAc-6P, in the absence (squares) and presence (circles) of 0.7 mM NanE.
511 The resultant kinetic parameters are presented in Table 2. The assay is described in
512 Materials and Methods.

513

514 **FIG. 5** The activity of NagB was measured as a function of HPr concentration 0-1.8
515 mM with 3 mM GlcN-6P in the presence (squares) and absence (circles) of 0.3 mM
516 NanE and in the presence of the effector GlcNAc-6P at 0.2 mM. Synergy of HPr and
517 NanE was observed at concentrations of HPr in excess of 0.9 mM

518

519 **FIG. 6** Synergistic effects in NagB protein-protein interactions (PPIs) possibly the
520 result of simultaneous binding of protein partners. (A-B) Activation of NagB by HPr
521 and NanE (A) as well as HPr and U-PII (B) was found to be synergistic. (C-E) The
522 binding of HPr to NagB does not sterically obstruct the binding of either NanE or U-
523 PII in the model, but the binding of NanE to NagB does sterically obstruct the
524 binding of U-PII (and vice versa). *Inset:* Binding sites for HPr, NanE, and U-PII on the
525 surface of NagB. Interacting residues are colored according to the binding partner,
526 and shared residues between NanE and U-PII are colored in grey. Note that the
527 majority of the NanE and U-PII interfaces on NagB overlap with each other. Residues

indicated by black lines are shared among all three NagB partners. The PDB IDs and chains used for NagB, HPr, and U-PII were 1FS5:A, 3CCD:A, 5L9N:A. 1FS5:A is a structure of the open, "R" conformation of NagB, and 5L9N:A is a structure of uridylylated PII. For NanE, the full-length Swiss Model Repository model based on the template 3IGS:A (79.7% sequence identity) was used.

TABLES

Table 1. The protein-protein interactome for NagB, suggesting that NagB interacts with numerous proteins in the *E. coli* cell.

Table 2. Kinetic parameters of NagB measured with respect to [GlcN-6P] in the presence and absence of PII or U-PII (in the presence of 0.4 mM GlcNAc-6P) at pH 8.1, and in the presence and absence of NanE at pH 7.8 (no GlcNAc6P) or at pH 6.8 with 0.2mM GlcNAc-6P. (U=μmol/min).

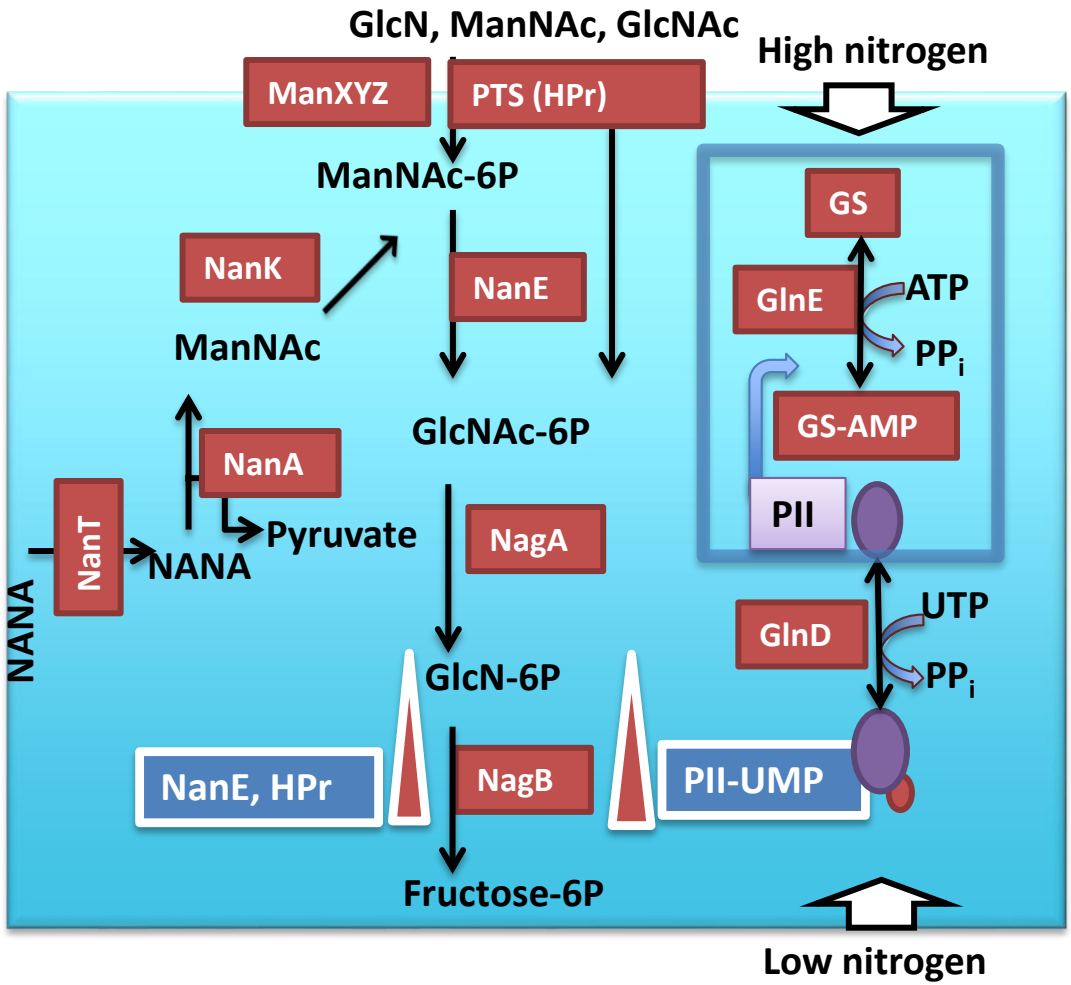

GlcN, ManNAc, GlcNAc
High nitrogen
ManXYZ
PTS (HPr)
ManNAc-6P
GS
NanK
NanE
GlnE
ATP
PPi
ManNAc
GS-AMP
GlcNAc-6P
NanA
PII
NanT
Pyruvate
NANA
NagA
UTP
GlnD
NANA
GlcN-6P
PPi
NanE, HPr
NagB
PII-UMP
Fructose-6P
Low nitrogen

FIG. 1

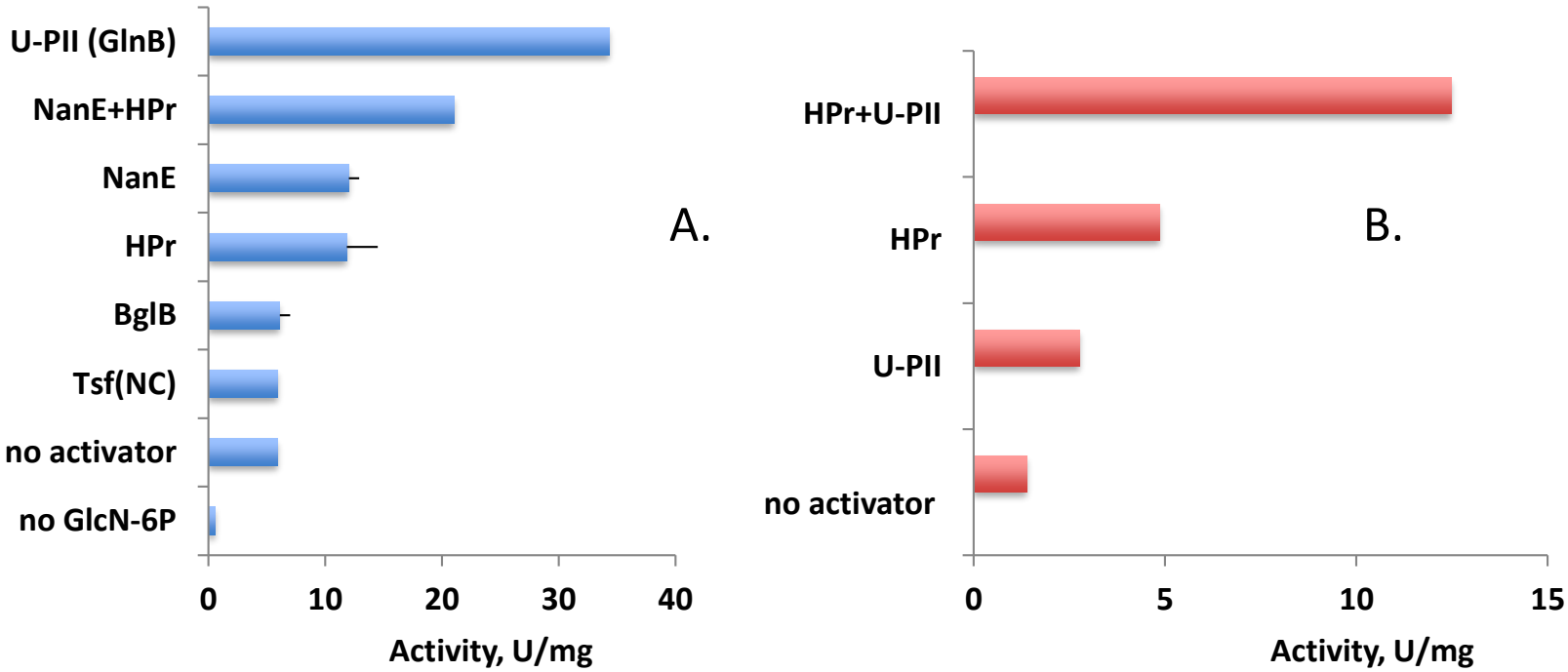

U-PII (GlnB)
NanE+HPr
NanE
HPr
BglB
Tsf(NC)
no activator
no GlcN-6P
0
10
20
30
40
Activity, U/mg
A.
HPr+U-PII
HPr
U-PII
no activator
0
5
10
15
Activity, U/mg
B.

FIG. 2

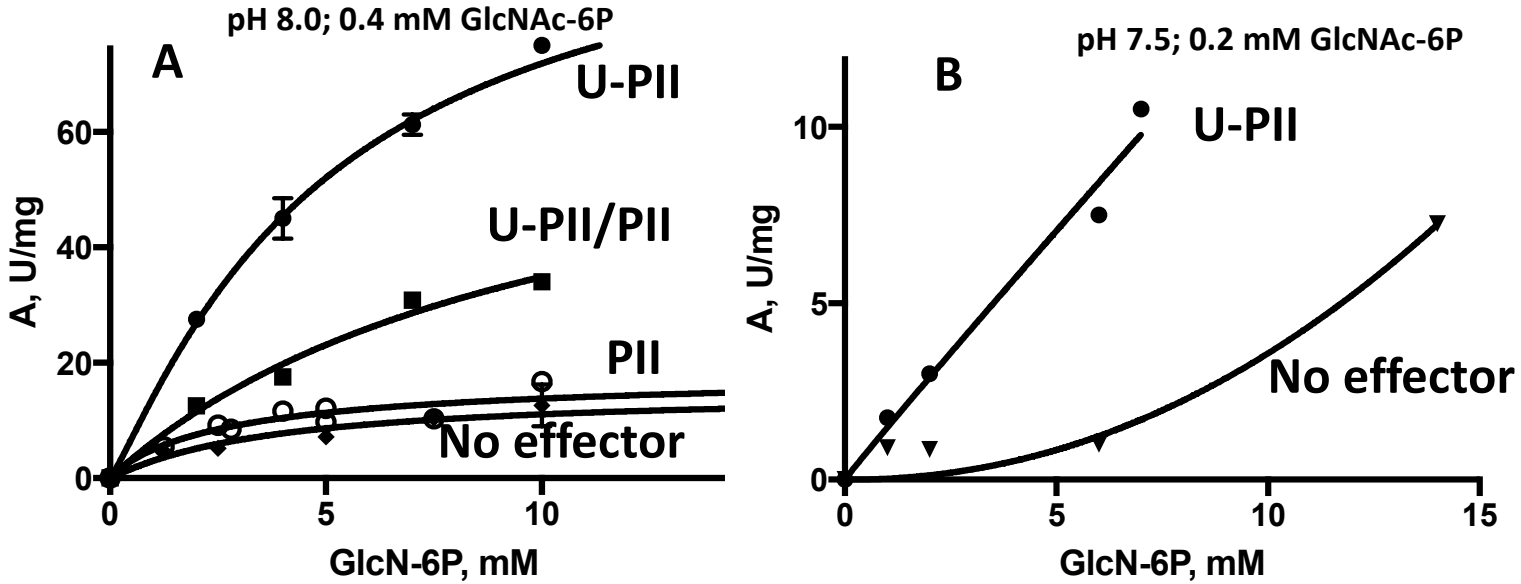

pH 8.0; 0.4 mM GlcNAc-6P
A
U-PII
U-PII/PII
PII
No effector
A, U/mg
GlcN-6P, mM
pH 7.5; 0.2 mM GlcNAc-6P
B
U-PII
No effector
A, U/mg
GlcN-6P, mM

FIG. 3

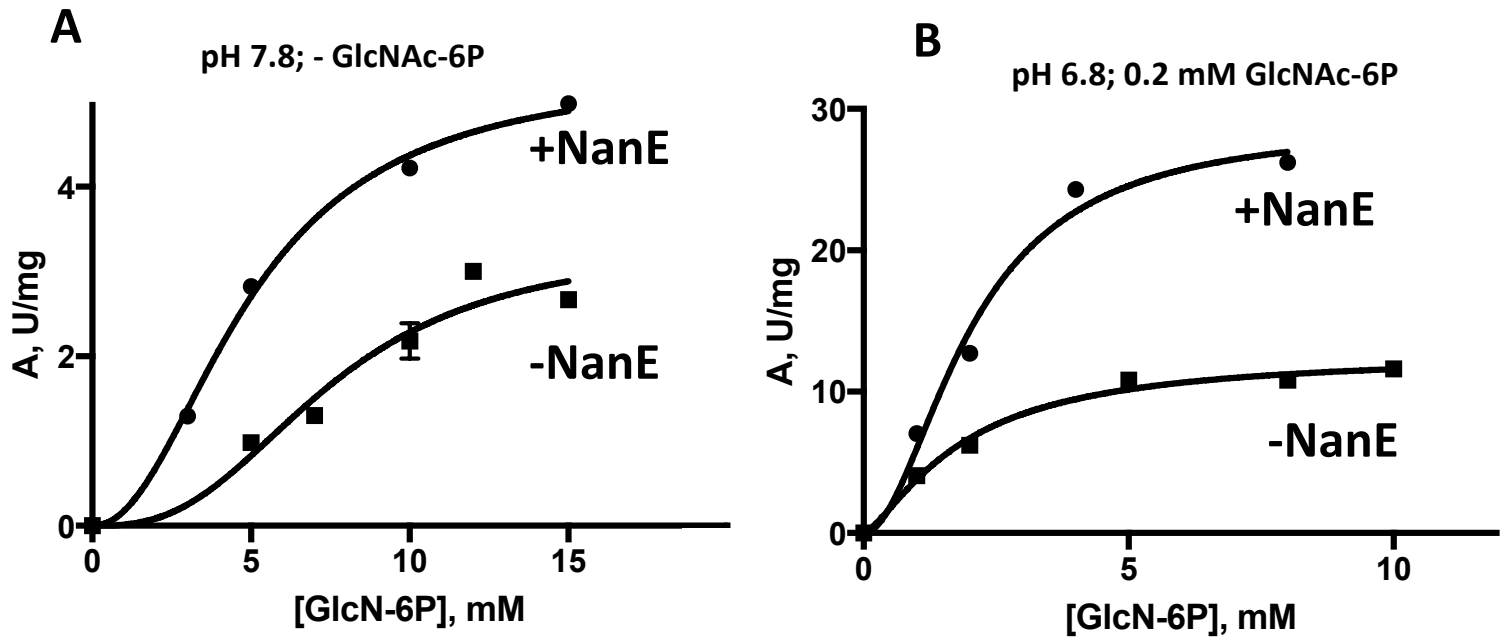

A
pH 7.8; - GlcNAc-6P
+NanE
-NanE
A, U/mg
0
2
4
0
5
10
15
[GlcN-6P], mM
B
pH 6.8; 0.2 mM GlcNAc-6P
+NanE
-NanE
A, U/mg
0
10
20
30
0
5
10
[GlcN-6P], mM

FIG. 4

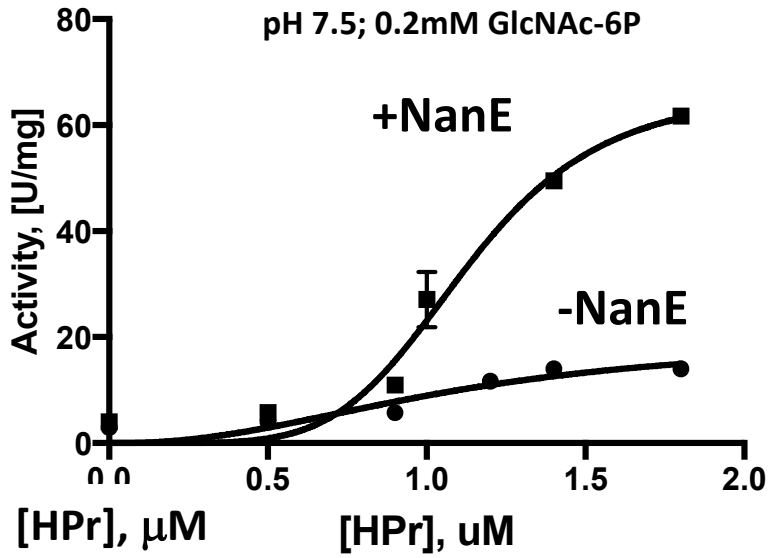
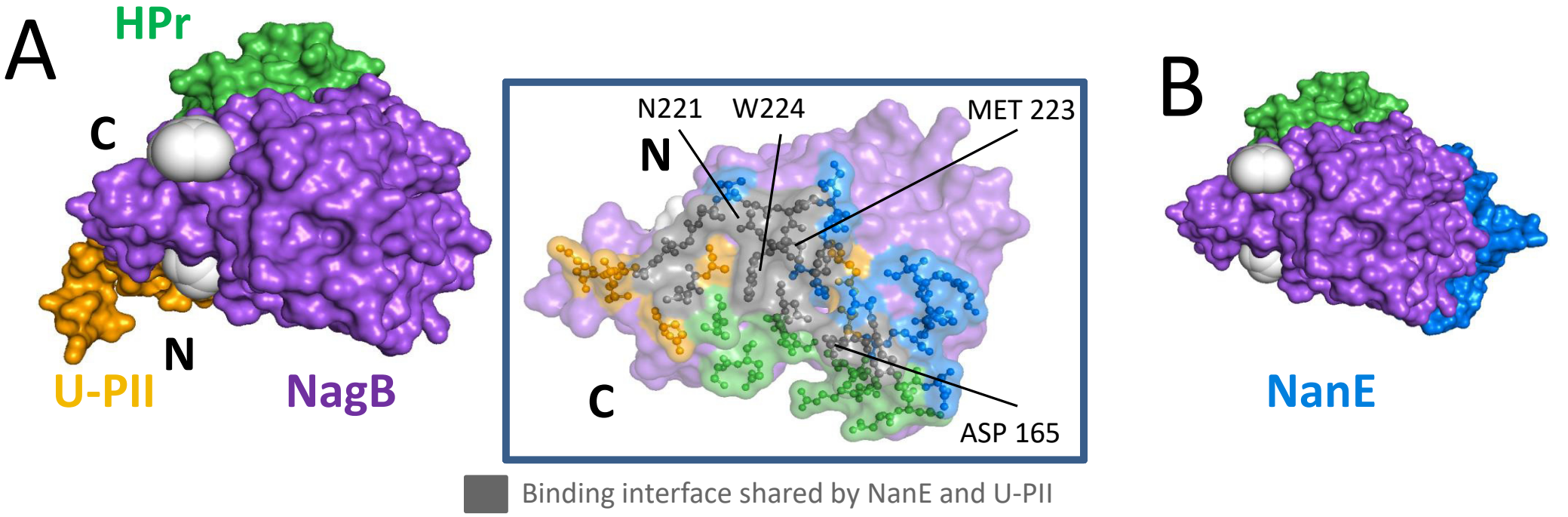

pH 7.5; 0.2mM GlcNAc-6P
+NanE
-NanE
Activity, [U/mg]
80
60
40
20
0
0.5
1.0
1.5
2.0
[HPr], μM
[HPr], uM

FIG. 5


A
HPr
C
N
U-PII
NagB
N221
W224
MET 223
N
C
ASP 165
B
NanE
Binding interface shared by NanE and U-PII
C
D
X
E