**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 Goodacre2, Mohan Babu3, Andrew Emili4, Peter Uetz2, and Milton H. Saier, Jr.1,\*

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

**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 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. NanE activates NagB in absence or presence of GlcNac-6P, but HPr and U-PII activation requires the presence of GlcNac-6P. 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, which suggests specific residues potentially involved in complex formation, provides a possible explanation. 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 the survival of bacteria in a competitive environment. NagB, glucosamine 6-phosphate deaminase, is essential for amino sugar utilization and allosterically regulated by N-acetyl-glucosamine 6-phosphate (GlcNAc-6P) and the histidine-phosphorylatable phosphocarrier protein HPr. We provide evidence in *E. coli* 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 the PII protein is known to be a central metabolic nitrogen regulator. Regulatory links between carbon and nitrogen metabolism are important for adaptation of metabolism to different growth conditions.

**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 microbiome member and model for Gram-negative bacteria is important for numerous biotechnological applications. *E. coli* tightly controls the utilization of amino sugars (3), which are excellent sources of both carbon and ammonia.

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 both 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*.* 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 (4) (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). NagA further deacetylates GlcNAc-6P to glucosamine 6-phosphate (GlcN-6P). GlcNAc-6P is also available from extracellular GlcNAc, transported into the cell by the bacterial PTS (5,6). Glucosamine 6-phosphate isomerase/deaminase, NagB, provides the last step in the amino sugar-specific 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 (7). Orthologs of this enzyme are present not only in bacteria, but also in mammals and other organisms. NagB is encoded in an operon with the *nagA* gene and is regulated by NagC (8), being induced when GlcN or GlcNac or other amino sugar is available in the medium.

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 the availability of extracellular PTS sugar substrates (9) including GlcNAc, ManNAc and GlcN.

NagB interactome data (Babu et al., manuscript submitted), reproduced in part 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 is activated in the presence of GlcNac-6P by the PII protein covalently modified by uridylylation, an indicator of nitrogen availability.

The uridylylated PII protein (U-PII), is generated by posttranslational modification under nitrogen limiting conditions involving the glutamine/-ketoglutarate ratio-sensing uridylyltransferase/uridylyl-removing enzyme, GlnD (10,11). 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. Bacteria regulate their metabolic activities according to the availability of carbon and nitrogen. The regulatory interdependence between different metabolic pathways has been considered (12), for example carbon metabolism is known be controlled not only by carbon derived signals, but also by the availability of nitrogen, sulfur and iron (13-15). Components of the phosphotransferase system (PTS) participate in regulatory interactions (Saier PMID: 8432744), resulting in the control of carbon and nitrogen metabolism (Refs PMID:20202847, Van Heeswijk WC, Westerhoff HV, Boogerd FC (2013) Nitrogen assimilation in *Escherichia coli*: putting molecular data into a systems perspective. Microbiol Mol Biol Rev 77(4):628–695,

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) and recently, the histidine-phosphorylatable phosphocarrier protein, HPr was shown to control the activity of glycolytic enzymes, energy balance and NagB by direct protein-protein interaction (9).

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 complex formation confirmed the possibility that the two proteins (HPr/U-PII or HPr/NanE) can simultaneous interact with NagB, although U-PII and NanE cannot. These observations are rationalized.

**Results**

**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 both the *E. coli* NagB and the non-orthologous deaminase/isomerase, NagB-II, from *Shewanella*, which belongs to the sugar isomerase protein family (16). 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). All of these proteins are involved in carbon and nitrogen metabolism. 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 5 mM of the substrate, GlcN-6P, NanE activated NagB, as shown in Fig. 2A. 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. The synergistic effect for the HPr and U-PII activation of NagB was measured at a 2mM concentration of substrate at 0.05 M U-PII (Fig 2B).

**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 was 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 proteins U-PII, PII, HPr have no activation effect on NagB in absence of 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 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 (17). 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. The activation of NagB by GlnD was tested with different effectors, and no activation or inhibition effect at the conditions described for the NagB assay was detected. GlnD was not purified from reaction mixture, no effect of the GlnD uridylylation reaction mixture for the NagB activity was noticed.

**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 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 2 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 (at 0.05 M) 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.

**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 involving 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 were either the largest or second-largest cluster in their respective docking outputs.

The grey balls in Fig.6 A are labelled with “N” and “C”, indicating that they represent the N- and C - termini of NagB. We have modified the figure and legend so it is clearer now. We have also made it clearer that NagB has been rotated in the inset, to show the NagB interaction interface. Note that the N- and C-termini are in different orientations in the inset than in Fig. 6 A. In the revision, we have also highlighted the intersubunit residues more explicitly, as this reviewer suggested.

The structure of NagB used was pdb id and chain 1FS5:B, which is the free active-site, ligand-bound, R conformer of a monomer of NagB. The R form seemed more appropriate than the unbound T form, since the structural modeling being performed was of NagB binding several protein partners, which were found in this study to promote activation of NagB. We do take into account the fact that functional NagB is a hexamer; it is a limitation of the modeling that we used the monomer. We attempted to generate a structure of the NagB hexamer using multi-body docking of six monomers of 1FS5:B; however, the output did not converge into distinct clusters and hence, the results were not conclusive. We have now discussed this in more detail in the discussion, including the possibility that the NagB hexamer forms an extended interface in which the inter-subunit cleft (as reviewer #2 indicates) assists in binding of the NagB partners.

With respect to whether complete information about known interacting residues was used, please note that the docking program used, HADDOCK, is a local refinement docking program and is optimized for finding the most biologically accurate conformation, but it requires an approximation of the interface as a guide. The highly-sensitive CPORT prediction server was used to make initial predictions prior to docking, and hence, we likely captured all known interacting residues, including these in the enzyme active sites. This may also be why it captured subunit interface residues. This is now discussed in much more detail in the Methods as well as the Discussion.

**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) (18-22). 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. An 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 PTS sugar substrates (9). 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 (9). 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 uridylylates 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, 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 on NagB or the NagB-HPr complex. 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 it can from HPr.

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 of simultanious U-PII/NanE simultaneous binding to NagB.

**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 using 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 (23), 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 (24). 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 (25) 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 (16). 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.

**Effect of different protein-protein interactions on 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 (26), 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 (27). *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 *E. coli* Zwf (NADPH-producing), under the conditions used, has no effect on NagB activity (data not shown). The enzyme with different cofactor specificity (NADH-producing) Zwf from *Leuconostoc mesenteroides* enzyme was used as couple enzyme (PMID:25246670) to test the effect of *E. coli* Zwf, and the NagB kinetics were the same for both coupled enzymes measurements.

**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.

**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 and 5L9N:A, respectively. 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.

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|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  | **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 |
| **Khalf, 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 |

**Table 2. A summary of the kinetics parameters characterizing the enzymatic consequences of the protein-protein interactions examined here.**

**FOOTNOTES**

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Abbreviations used are: NagB, Glucosamine 6-phosphate isomerase/deaminase; HPr, histidine-phosphorylatable phosphocarrier protein; PTS, phosphotransferase system;

**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 Vmax. 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, phosphotransferase system; ManXYZ, the mannose PTS system; NanT, the NANA transporter; NanK, ManNAc kinase; GS, glutamine synthetase; PII (GlnB), nitrogen regulator; GlnD, uridylyltransferase/uridylylase for the PII protein, GlnE; adenylyltransferase/adenylase

**FIG. 2** Protein-protein interaction-dependent activation of NagB in the presence of a non-saturating concentration of the allosteric effector, GlcNAc-6P (0.4 mM). A. Activity was measured at 5 mM GlcN-6P in the presence of NanE (0.7M), PII (GlnB) (1M) and /or HPr (0.5 M). BglB (0.2M) and Tsf (2M) were included as negative controls. B. Activity was measured at 2 mM GlcN-6P in the presence of HPr (0.5M), U-PII (0.05M) or a combination of the same concentrations of HPr and U-PII, at pH 7.5.

**FIG. 3** Allosteric activation of NagB by the uridylylated PII protein (U-PII). A. The kinetics were measured as a function of the GlcN-6P concentration (0 to 10 mM) in the presence of the allosteric effector, 0.4 mM GlcNAc-6P in the absence (diamonds) or the presence (squares) of 0.4 mM partially uridylylated U-PII (squares), 0.4 mM fully uridylylated U-PII (filled circles) or 0.6 mM free PII (open circles), all at pH 8. The assay is described in Materials and Methods. B. Steady-state kinetics of NagB measured as a function of the GlcN-6P concentration (0 to 14 mM) in the presence of the allosteric effector, 0.2 mM GlcNAc-6P in the absence (triangles) or presence (filled circles) of 0.2 mM U-PII, at pH 7.5. One unit of activity = 1 mMol of product formed/mg protein/min.

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

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

**FIG. 6** Synergistic effects in NagB protein-protein interactions (PPIs), possibly the result of simultaneous binding of protein partners.(A-B)Activation of NagB by HPr and NanE (A) as well as HPr and U-PII (B) was found to be synergistic. (C-E) The binding of HPr to NagB does not sterically obstruct the binding of either NanE or U-PII in the model, but the binding of NanE to NagB does sterically obstruct the binding of U-PII (and vice versa). *Inset:* Binding sites for HPr, NanE, and U-PII on the surface of NagB. Interacting residues are colored according to the binding partner, and shared residues between NanE and U-PII are colored in grey. Note that the 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, respectively. 1FS5:A is a structure of the open, “R” conformation of NagB, and 5L9N:A is a structure of uridylated PII. For NanE, the full-length Swiss Model Repository model based on the template 3IGS:A (79.7% sequence identity) was used.

**TABLES**

**Table1.** 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=mol/min).