**Global regulation of energy metabolism by the phosphocarrier protein of the phosphotransferase system, HPr, in Escherichia coli.**

Irina A. Rodionova\*, Zhongge Zhong, and Milton H. Saier, J.\*

Department of Molecular Biology, Division of Biological Sciences, University of California at San Diego, La Jolla, CA 92093 USA

\*To whom correspondence should be addressed. Email: [irodionova@ucsd.edu](mailto:irodionova@ucsd.edu); Email: msaier@ucsd.edu

**Abstract**

We provide evidence that the phosphocarrier protein, HPr, an essential component of the bacterial phosphotransferase system (PTS), interacts with a large number of proteins in *Escherichia coli*. We demonstrate HPr-dependent allosteric regulation of the activities of pyruvate kinase (PykF, but not PykA), phosphofructokinase (PfkB, but not PfkA), glucosamine 6-phosphate deaminase (NagB) and adenylate kinase (Adk). HPr exists in the cell either phosphorylated on a histidyl residue (HPr-P) or non-phosphorylated (HPr). Activation of PykF occurs only by non-phosphorylated HPr with a decrease in its Khalf for PEP of 10 fold (from 4.3 mM to 0.29 mM), thus influencing glycolysis. Activation of PfkB by HPr, but not HPr-P, results from a decrease in the Khalf for fructose-6-P which likely influences gluconeogenesis. Activation of NagB shown for HPr is important for utilization of aminosugars. Allosteric inhibition of Adk activity by HPr-P, but not HPr, was also demonstrated, thus allowing HPr to regulate the cellular energy charge. These observations suggest that HPr serves as a direct interaction global regulator of carbon and energy metabolism and probably of other physiological processes in enteric bacteria.

**Introduction**

The **Enterobacteriaceae** comprise a large family of Gram-negative proteobacteria that includes many human, animal and plant pathogens. These include *Escherichia*, *Salmonella, Yersinia,* *Klebsiella, Shigella, Erwinia*, (*Dickeya*), *Pantoea* and *Pectobacterium* ([Toth *et al.*, 2006](#_ENREF_54); [Charkowski *et al.*, 2012](#_ENREF_11); [Malnoy *et al.*, 2012](#_ENREF_28); [Porcheron *et al.*, 2016](#_ENREF_34)). In these organisms and many others, the histidine phosphorylatable phosphocarrier protein, HPr, is an essential constituent of the sugar transporting phosphoenolpyruvate (PEP):sugar phosphotransferase system (PTS) ([Saier *et al.*, 1996](#_ENREF_43); [Barabote and Saier, 2005](#_ENREF_7)). HPr energizes PTS Enzyme II complexes ([Reichenbach *et al.*, 2007](#_ENREF_39)) and phosphorylates the *E. coli* central regulatory protein, IIAGlc (Crr) ([Saier and Roseman, 1976](#_ENREF_46)), as well as the PEP-dependent dihydroxyacetone kinase (DhaK) ([Gutknecht *et al.*, 2001](#_ENREF_18); [Garcia-Alles *et al.*, 2004](#_ENREF_16)). HPr has also been shown to interact with and regulate the *E*. *coli* glycogen phosphorylase ([Seok *et al.*, 1997](#_ENREF_50); [Seok *et al.*, 2001](#_ENREF_49)). However, interactions of HPr with other cellular constituents for purposes of regulation are not well established.

Recently obtained *E. coli* interactome data (Babu et al. manuscript in preparation) have suggested that other carbohydrate metabolic enzymes interact with HPr (Table 1). Proposed targets of HPr regulation include the critical glycolytic enzymes: 6-phosphofructokinase II (PfkB), pyruvate kinase F (PykF), glucosamine 6-phosphate deaminase (NagB) (Fig. 1) and 3-deoxy-D-manno-octulosonate-8-phosphate synthase (KdsA). HPr also appears to interact with adenylate kinase (Adk), which reversibly converts two molecules of ADP to ATP + AMP ([Schrank *et al.*, 2013](#_ENREF_47)). Proteins already known to interact with HPr include PTS proteins, DhaK and Crr. The iron storage and detoxification protein, bacterioferritin (Bfr) is another interesting potential target ([Wong *et al.*, 2015](#_ENREF_59)). HPr also appear to interacts with the major cold shock protein, CspA, an RNA chaperone protein ([Phadtare and Inouye, 2008](#_ENREF_31); [Phadtare and Severinov, 2010](#_ENREF_32)), and the CysK subunit of cysteine synthase, an O-acetylserine sulfhydrolase ([Campanini *et al.*, 2015](#_ENREF_10)). Summarizes potential interactions of HPr with a variety of proteins is represented in Table 1.

The data presented in Table 1 suggest that HPr plays a role in ribosome-dependent protein biosynthesis ([Achenbach and Nierhaus, 2014](#_ENREF_1)). Interactions relevant to this process include those with at least five proteins: chain elongation factor, EF-Ts (the Tsf protein), which influences the rate of translational elongation ([Kraal *et al.*, 1999](#_ENREF_24)), the anti-σ70 factor, Rsd, that under certain conditions controls translational initiation ([Sharma and Chatterji, 2010](#_ENREF_52); [Park *et al.*, 2013](#_ENREF_30)), two ribosome processing proteins, RimM and RimP ([Shajani *et al.*, 2011](#_ENREF_51)), and Hpf, a ribosome hibernation-promoting factor ([Vila-Sanjurjo, 2008](#_ENREF_56)).

Kim et al. showed that one of the three pyruvate kinases in *Vibrio vulnificus* PykA is activated by the non-phosphorylated form of HPr ([Kim *et al.*, 2015](#_ENREF_22)). The PTS, which catalyses the first step of glycolysis, thus, also stimulates the final step in the presence of exogenous glucose through the direct interaction of HPr with the C-terminal domain of *Vibrio* PykA. Kim et al. examined the *E. coli* PykA, but regulation could not be demonstrated ([Kim *et al.*, 2015](#_ENREF_22)). *E. coli* PykF was not tested.

Many Enterobacteriaceae, including *E. coli,* have two isoforms of pyruvate kinase, PykA and PykF. Pyruvate kinase generates ATP from ADP and PEP, the last step in the glycolytic pathway, a step that is irreversible under physiological conditions. PykF is an allosterically regulated enzyme and exhibits sigmoidal kinetics toward PEP. Allosteric regulation by fructose 1,6-bisphosphate (FBP) reflects the central position of PykF in cellular metabolism ([Valentini *et al.*, 2000](#_ENREF_55)). The global transcriptional regulator, Cra (FruR), controls *pykF* transcription in *E. coli* ([Ramseier *et al.*, 1993](#_ENREF_37); [Ramseier *et al.*, 1995](#_ENREF_36); [Saier and Ramseier, 1996](#_ENREF_44); [Ravcheev *et al.*, 2014](#_ENREF_38)). Two isoforms of phosphofructokinase exist in *E. coli*, PfkA and PfkB (Fig.1). PfkB was shown to be inhibited by MgATP at low concentrations of Fru-6P, and this regulation is important for gluconeogenesis ([Sabnis *et al.*, 1995](#_ENREF_41)).

Adenylate kinase (Adk) is a ubiquitous cellular energy (nucleotide) homeostasis enzyme, catalyzing reversible AMP phosphorylation using ATP for ADP production. The activity of Adk is allosterically inhibited by AMP. Adk has 3 major domains: a CORE domain, an ATP-binding domain, and an AMP-binding domain. The enzyme is known to transit between open and closed conformational states ([Ping *et al.*, 2013](#_ENREF_33)).

Glucoseamine-6-phosphate deaminase (NagB) is a catabolic enzyme for the utilization of N-acetylglucoseamine (GlcNAc), N-acetylmannosamine (ManNAc), N-acetylneuraminic acid (NANA) and glucosamine(GlcN) (Fig. 1). GlcNAc, ManNAc and GlcN are PTS sugars ([Postma *et al.*, 1993](#_ENREF_35)) in E. coli so that their uptake occurs concomitantly with their phosphorylation, which produces intracellular GlcNAc6P, ManNAc6P and GlcN6P, respectively. GlcNAc6P is first deacetylated by the NagA to GlcN6P, which is then subject to deamination and isomerization by the NagB (GlcN6P deaminase/isomerase), resulting in production of ammonia and fructose 6-phosphate, which enters the glycolytic pathway. The enzyme is allosterically activated by GlcNAc6P binding, known as heterotropic allosteric activation ([Alvarez-Anorve *et al.*, 2011](#_ENREF_4)). GlcN6P deaminase has two extreme structural states, with high substrate affinity (the R state) and low affinity for GlcN6P (the T state). The GlcNAc6P binding sites are present at the subunit interfaces of the hexamer, and as a consequence of GlcNAc6P binding the enzyme transitions to the R state. Thus the allosteric transition activates the enzyme, increasing its apparent affinity for GlcN6P (Km) without a change in the catalytic constant (kcat) ([Altamirano *et al.*, 1995](#_ENREF_3))

In this study we aspired to test and understand the types of HPr regulation by biochemically characterizing the effects of HPr and HPr-P on the activities of PykF, PfkB, NagB and Adk in *E*. *coli*. The kinetics measured in the presence of HPr depend on its state of phosphorylation; PykF, PfkB and NagB are activated by HPr but not HPr-P, while Adk is inhibited by HPr-P but not HPr. HPr is expected to be in the non-phosphorylated form when a PTS sugar such as glucose or fructose is present in the extracellular medium, and in the phosphorylated form in the absence of a PTS sugar ([Deutscher *et al.*, 2014](#_ENREF_12); [Kim *et al.*, 2015](#_ENREF_22)). Thus, the phosphorylation state of HPr can be considered as an indicator of exogenous carbon and energy availability.

**Results.**

***Non-phosphorylated HPr activates PykF.***

*E. coli* PykF activity was measured following the decrease in absorbance at 340 nm resulting from the oxidation of NADH in a coupled assay involving lactate dehydrogenase (LDH) (see Materials and Methods). This assay is based on the ADP-dependent conversion of PEP to pyruvate by PykF, and further reduction of the pyruvate formed to lactate by LDH. To investigate the specific regulation of PykF, PfkB, NagB and Adk activities by HPr, the proteins: Enzyme I (EI), PykF, PfkB, NagB, Adk and HPr were purified to homogenity.

Steady state kinetics for PykF were measured with respect to PEP concentration in the absence and presence of 1 M HPr (Fig. 2A). Parameters derived from the kinetics are presented in Table 2. The presence of HPr caused the Khalf for PEP to decrease 10-fold. Thus, HPr influenced the affinity of PykF for PEP and accordingly activated PykF at low PEP concentrations (Fig. 2A). To determine the effects of either HPr-P or HPr on the activity of PykF, steady state kinetics were measured at a fixed PEP concentration by titration of HPr. The phosphorylated form was obtained by pre-reaction with PEP and EI for 30 min at 37oC. HPr-P had no effect on PykF activity, but HPr showed a positive effect, with a measured Kd for HPr of 0.1 μM (Fig 2B). The best effect observed with HPr at a concentration of 1 M depended on the presence of 100 M ZnSO4. Titration with zinc at an HPr concentration of 1 μM is presented in Fig 1C.

A proteoimic analysis was conducted using LC-MS/MS for PykF co-purified with HPr. By contrast, no HPr co-purified with PfkB under same conditions (Table S1), presumably reflecting the relative affinities of the two enzymes for HPr.

***Non-Phosphorylated HPr activates Phosphofructokinase (PfkB).***

*E. coli* PfkB activity was measured using the coupled assay described in Materials and Methods. PfkB-dependent fructose-6P (Fru-6P) phosphorylation converts ATP to ADP, and ADP is used by PK/LDH to convert PEP to pyruvate and further to lactate following the decrease in absorbance at 340 nm resulting from the oxidation of NADH in a coupled assay. PfkB activity was previously known to be allosterically inhibited by ATP in the presence of 50 mM KCl ([Babul, 1978](#_ENREF_6); [Cabrera *et al.*, 2010](#_ENREF_9)). To evaluate the effect of free HPr, steady-state kinetics were measured in the presence of 2.2 M HPr. Although HPr showed no effect on the Vmax or the Hill coefficient (h), the presence of HPr led to a 6-fold decrease in the Khalf for Fru-6P. This result implies that HPr regulates PfkB activity by increasing the affinity of PfkB for its substrate, Fru-6P. It is important to emphasize that the non-phosphorylated form of HPr activated PfkB in the presence of 50 mM KCl and 1mM ATP, decreasing the Khalf for Fru-6P from 0.49 to 0.08 mM (Fig. 3A; Table 2). HPr did not appear to completely antagonize inhibition by ATP (Fig 3B), but KCl strengthened the inhibitiory effect of ATP and was required for activation by HPr.

To establish the effect of HPr phosphorylation on PfkB activity, HPr was fully phosphorylated with PEP and EI. To determine if HPr-P affected PfkB activity, EI (10 g/ml), PEP (1 mM) and different concentrations of HPr-P were added in the PfkB assay reaction. The addition of HPr-P, up to 3 M, did not result in activation or inhibition, although HPr saturates PfkB for activation at about 2.2 M (Fig 3B).

***Non-Phosphorylated HPr activates GlcN6P deaminase (NagB).***

*E. coli* NagB activity was measured following the increase in absorbance at 340 nm resulting from the reduction of NADP in a coupled assay involving phosphoglucose isomerase (Pgi) and glucose 6-phosphate dehydrogenase (Zwf) (see Materials and Methods). This assay is based on the conversion of GlcN6P 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. The activation of NagB by GlcNAc6P was shown in the presence and absence of HPr (Fig4A). These kinetics were measured using 0.2 mM GlcNAc6P in the presence and absence of 2M HPr at pH 8 and without GlcNAc6P at pH 6.5(Fig4B). A substantial increase in activity was observed, particularly at low concentrations of the substrate GlcN6P at pH 6.5 without, GlcNAc6P, and at pH 8 with GlcN6P.

***Phosphorylated HPr inhibits Adk.***

The activity of Adk was measured in a pyruvate kinase (PK) and lactate dehydrogenase (LDH) coupled assay as described in Materials and Methods. The activity of Adk was followed by measuring a decrease in the absorbance at 340 nm resulting from the oxidation of NADH when AMP and ATP were incubated with Adk to generate ADP. PEP and ADP then react to generate pyruvate and ATP, and the pyruvate formed is reduced to lactate, measuring the decrease in the absorption at 340 nm when NADH is oxidized to NAD+. The kinetics were measured in the presence and absence of 1M HPr-P or HPr (Fig 5). The results revealed the inhibitory effect of the phosphorylated form of HPr on Adk (Fig 5A), but HPr itself was without effect.

Substantial inhibition was observed at concentrations of AMP less than 1 mM. The titration of HPr-P shown in Fig. 4B, revealed that saturation occurred at a concentration of 1.0 M; the Ki for HPr-P binding was 0.1 M. Table 2 presents the changes in the kinetic parameters upon HPr-P binding. The Khalf increased more than 7-fold, and as shown in Fig. 5B, the inhibition of Adk is substantial, especially at low concentrations of AMP.

**Discussion**

*E. coli* HPr interactome analyses (Table 1) suggested direct interactions with glycolytic enzymes - PykF and PfkB and a key enzyme for aminosugar catabolism that feeds the glycolysis, NagB, as well as adenylate kinase, all possible targets of regulation by HPr. Pyruvate kinase, PykF, plays a central role in glycolysis, producing ATP in the last glycolytic reaction, by converting PEP and ADP to pyruvate and ATP (Fig.1). The data presented show activation of PykF by the non-phosphorylated form of HPr with a decrease in the Khalf of around 10-fold.

Phosphofructokinase II, PfkB, is known to be allosterically inhibited by MgATP in the presence of physiological concentrations of KCl, and this inhibition appears to be important for the regulation of gluconeogenesis ([Torres *et al.*, 1997](#_ENREF_53)). We showed that the non-phosphorylated form of HPr combats ATP inhibition within the concentration range of ATP from 0.1 to 3 mM (Fig2). The flux of intracellular glucose-6P from exogenous glucose inhibits gluconeogenesis in the cell, and PfkB regulation by MgATP is partially abolished by the interaction with HPr. It seems from the data, however, that HPr activates by a mechanism that is at least partially independent of allosteric MgATP inhibition.

Glucosamine 6-P deaminase NagB, feeds directly into glycolysis and has been shown to be allosterically activated by GlcNac6P (**Calcagno**). We found that HPr increases the activity of enzyme by decreasing the apparent affinity of the enzyme for its substrate (Fig. 3). Thus in all the glycolytic enzymes HPr affects the enzyme activity by changing the Khalf without appreciably altering the Vmax. Moreover in all this cases the phosphorylated form of HPr, HPr-P, was without affect, suggesting that HPr binds to these enzymes on its phosphorylatable phase as is true for the interaction of HPr with all of the PTS enzymes, EI and the EII proteins (PMID:22593574).

Adenylate kinase is an essential enzyme that catalyzes the reversible conversion of AMP and ATP to 2 molecules of ADP(Fig.1). The substrate-bound closed conformation of this enzyme is regulated by ATP (Fig. 5). Our results demonstrated substantial inhibition of adenylate kinase activity by HPr-P at low AMP concentrations. They indicated that in the absence of an exogenous PTS sugar substrate (i.e., D-glucose, D-N-acetylglucosamine, D-mannose, D-mannitol etc.), the catalytic reaction producing ADP is inhibited. Thus the presence of an exogenious PTS sugars regulaes all four enzymes studied here in a coordinated fashion.

The key conserved aspects of regulation by HPr are:

(1) PykF, PfkB and NagB catalyze a glycolytic reactions and PfkB additionally regulates gluconeogenesis. These enzymes are all activated by HPr. (2) HPr is present in the cell in the phosphorylated (HPr-P) or non-phosphorylated (HPr) form depending on the presence or absence of a sugar substrate of the PTS in the medium ([Deutscher *et al.*, 2014](#_ENREF_12); [Kim *et al.*, 2015](#_ENREF_22)). Such sugars, when present, give rise to the dephospho form of the protein due to sugar phosphorylation, but when exogeneous PTS sugars are absent, HPr-P should predominate. (3) The first reaction of glycolysis involves sugar uptake and phosphorylation mediated by the PTS. The concentration of the phosphorylated form of HPr decreases in the presence of a PTS substrate ([Postma *et al.*, 1993](#_ENREF_35); [Hogema *et al.*, 1998](#_ENREF_19)). Accordingly, the free HPr concentration increases. (4) HPr activates NagB, PfkB and PykF by reducing the Khalf for these enzymes for their substrates, and the overall glycolytic flux should therefore increase with availability of an exogenous PTS sugar. All three glycolytic enzymes are known to be rate-limiting ([Saier, 1987](#_ENREF_42)). (5) The biosynthetic pathway producing UDP-GlcNAc for incorporation into cell wall components involves the glmS, glmM, and glmU gene products and utilizes the cytoplasmic GlcN6P pool. NagB redirects

the flux of GlcN6P utilization to the glycolytic pathway and is thus important and tightly regulated by two factors GlcNAc6P an intermediate of the catabolic pathway and the main product of PTS system and a PTS sensor HPr for GlcNAc, ManNAc, NANA and GlcN6P. In the allosteric regulation of NagB by HPr and GlcNAc6P, catabolism of aminosugars is effectively coordinated with other metabolic reactions occuring in the *E. coli* cell.

In a PykF- mutant, the flux through the glycolytic pathway was reduced, and that through the oxidative pentose phosphate (PP) pathway was increased ([Al Zaid Siddiquee *et al.*, 2004](#_ENREF_2)). This was evident by the corresponding enzyme activities and the increases in the cytoplasmic concentrations of phosphoenolpyruvate, glucose-6-phosphate and 6-phosphogluconate ([Al Zaid Siddiquee *et al.*, 2004](#_ENREF_2)). PykF produces pyruvate - a key intermediate in several catabolic and biosynthetic reactions. Several metabolic routes can furnish this compound, but the major route is through the activated PykF. Thus, activation of PykF by HPr should lead to global changes in *E. coli* carbon and energy metabolism.

Adenylate kinase allows the conversion of AMP and ATP into two molecules of ADP, and thus facilitates the distribution of the three adenosine phosphates, AMP, ADP and ATP, to allow homeostatic control of nucleotides and monitor the cellular energy charge ([Wujak *et al.*, 2015](#_ENREF_61)). The kinetics of adenylate kinase crucially changes in the presence of HPr-P, in response of the *E. coli* cell to the absence or presence of exogenous sugars. Thus, all four enzymes, NagB, PfkB, PykF and Adk increase in activity when PTS sugars are available. To what extent these regulatory mechanism are operative in other enteric bacteria and their more distant relatives has yet to be determined.

**Materials and Methods**

**Protein purification**. Recombinant proteins containing an N-terminal His6 tag were overexpressed in E. coli and purified using Ni2+-chelating chromatography. The E. coli OE strains, overexpressing PykF, PfkB, Adk, PtsH or PtsI from the ASKA collection ([Kitagawa *et al.*, 2005](#_ENREF_23)) 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 ([Rodionova *et al.*, 2013](#_ENREF_40)). Briefly, cells were harvested and resuspended in 20 mM HEPES buffer, pH 7, containing 100 mM NaCl, 2 mM β-mercaptoethanol, and 0.03 % Tween 20 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, Tris-HCl buffer (pH 8) was added to the supernatant to a final concentration of 50 mM. The supernatant was then loaded onto an Ni-NTA agarose minicolumn (0.2 ml) from Qiagen Inc. (Valencia, CA). After bound proteins were washed with At-buffer containing 50 mM Tris-HCl buffer (pH 8), 0.5 M NaCl, 5 mM Imidazole and 0.3 % Tween 20, they were eluted with 0.3 ml of the same buffer supplemented with 250 mM imidazole. Protein size, expression level, distribution between soluble and insoluble forms, and extent of purification were monitored by SDS-PAGE. All proteins except HPr were obtained with high yield (>1 mg) and purity (80 to 90%). The *E. coli* OE strain, used for HPr protein purification, was grown in 2L of LB medium at 37 oC, induced by 0.6 mM IPTG at 24 oC and harvested after 12 hours of shaking. Cells were resuspended in the same lysis buffer as described previous, and after lysis, sonication, and centrifugation, the insoluble fraction was resuspended in At-buffer containing 7M Urea, 0.3% Brij and 25 mM Imidazole. Inclusion bodies were dissolved, and after sonication and centrifugation, they were purified on an NTA agarose column. The bound protein was washed with 7M Urea At-buffer, HPr was refolded by washing the column with At-buffer containing 0.3% Brij, and it was eluted with the same buffer containing 300 mM imidazole on an FPLC system. The buffer was changed to At-buffer by dialysis. Protein concentrations were measured using the Bradford assay kit (Biorad).

**Cloning *nagB* into pMST3**

The *nagB* gene, encoding Glucosamine-6-phosphate deaminase, was PCR amplified from *E. coli* BW25113 chromosome using oligos nagB-Bam-F : ataggatccagactgatccccctgactaccgctgaac and nagB-Sal-R ctcgtcgacttacagacctttgatattttctgcttc.

The products were gel purified, digested with *BamH*I and *Sal*I, then cloned into the pSMT3 vector digested with the same enzymes. 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 immediately to the 3’ end of the SUMO gene (without stop codon) encoding the SMT3 tag. The expression of “SUMO:*nagB*” is under the control of a T7 promoter. The SMT3 tag present in the fusion protein was removed by a Ulp1 Sumo protease. The resultant NagB enzyme, has serine residue instead of first residue - methionine.

**Enzyme assays.** Activities of the purified recombinant *E. coli* Adk, PykF, NagB and PfkB were routinely assayed in a cuvette at 37°C using the standard enzymatic coupling assays as described previously (). Briefly, the ATP-dependent adenylate kinase activity was assayed by coupling the formation of ADP to the oxidation of NADH to NAD+ via PK and LDH with continuous monitoring at 340 nm in a Beckman reader. Adenylate kinase (1 ng) was added to 100 μl of a kinase reaction mixture containing 50 mM Tris-HCl buffer (pH 7.5), 10 mM MgSO4, 1.2 mM ATP, 1.2 mM PEP, 0.3 mM NADH, 1.2 U of PK, 1.2 U of lactate dehydrogenase (LDH), 2mM DTT, and 0-2 mM AMP substrate. To determine the effect of HPr on PykF, PfkB, NagB or Adk activity, 0-2 M HPr-P or HPr was added to an assay mixture. HPr was phosphorylated in the assay mixture containing 100 mM TRIS, pH 8, 2 mM DTT, 8 mM PEP, 10 mM MgCl2 and EI and incubated for 40 min at 30 oC. Subsequently, up to 3 M of HPr-P, 10 g/ml of EI and 8 mM PEP (final concentrations) were added to the assay mixture. The observed reaction rates (calculated using an NADH extinction coefficient of 6220 M−1 cm−1) were compared to those for the two sets of control samples: one control without the tested enzyme and another without AMP. The Khalf and Vmax values were determined using GraphPad Prism software.

PfkB activity was assayed in the same format as Adk; the substrate, fructose-6-phosphate instead of AMP, was added to the mixture. PfkB (24 ng) was added to 100 μl of a reaction mixture containing 50mM KCl and reaction rate were compared to controls in which fructose-6-phosphate was absent.

Pyruvate kinase (PykF) activity was tested using a coupled assay with LDH. PykF (15 ng) was added to 100 μl of a reaction mixture containing 200 mM Tris-HCl buffer (pH 7.5), 10 mM MgSO4, 1.5 mM ADP, 0-8 mM PEP, 0.3 mM NADH, 0.2 M KCl, 100 M ZnSO4, 5 mM phosphate and 1.2 U of LDH. Titration of HPr was similarly assayed, but for measuring the effect of HPr-P on PykF activity, 2mM DTT, 2 mM PEP and 10 g/ml EI were also added to the mixture.

GlcN6P deaminase activity was assayed by coupling the formation of fructose 6-phosphate to the reduction of NADP with monitoring at 340 nm in a Beckman reader. NagB (20 ng) was added to 100 μl of a reaction mixture containing 100 mM Tris-HCl buffer (pH 8), 5 mM MgSO4, 0-15mM GlcN6P, 2 mM NADP, 1.2 U of Pgi, 1.2 U of Zwf, 5mM phosphate, and 10M ZnSO4. To determine the effect of HPr on NagB activity, 0-2 M HPr was added to the assay mixture. The observed reaction rates (calculated using an NADPH extinction coefficient of 6220 M−1 cm−1) were compared to those for the control sample.

**Proteomic analyses of proteins interacting with PykF.**

For the pull-down assay, PykF or PfkB *E. coli* OE cell extracts with phosphate added to 15 mM was loaded onto Ni-NTA columns, and after washing with 1ml At-buffer, it was eluted with the same buffer containing 300 mM imidazole. The proteomic analyzes were done using LC-MS/MS with the facility at Sanford Burnham Prebys Medical Discovery Institute. For the sample preparation, protein concentration was determined using bicinchoninic acid (BCA) protein assay (Pierce, Waltham, MA). Samples were digested using a modified Filter-aided Sample Preparation (FASP) protocol ([Wisniewski *et al.*, 2009](#_ENREF_58)). In brief, each sample was transferred to a 10-kDa molecular weight cutoff filter (Millipore, MA, USA), then washed three times with UA buffer (8M urea, 50 mM ammonium bicarbonate), and cysteine disulfide bonds were reduced with 5 mM dithiothreitol (DTT) at 30°C for 60 min followed by cysteine alkylation with 15 mM iodoacetamide (IAA) for 30 min. Following alkylation, the samples were washed 2 times with UB buffer (1 M urea, 50 mM ammonium bicarbonate). The samples were finally subjected to overnight digestion with mass spec grade Trypsin/Lys-C mix (Promega, Madison, WI). The resulting peptides were eluted off the filter by centrifugation. In addition, the filter was washed twice with 15% acetonitrile to increase peptide recovery. The digested proteins were desalted using AssayMap C18 cartridges mounted on a BRAVO liquid handling system (Agilent, Columbia, MD), and the organic solvent was removed in a SpeedVac concentrator prior. Dried samples were reconstituted with 2% acetonitrile, 0.1% formic acid and analyzed by LC-MS/MS using a Proxeon EASY nanoLC system (Thermo Fisher Scientific) coupled to an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific). Peptides were separated using an analytical C18 Acclaim PepMap column, 0.075 x 250 mm, 2µm particles (Thermo Scientific) in a 180-min linear gradient of 2-28% solvent B at a flow rate of 300 nL/min. The mass spectrometer was operated in positive data-dependent acquisition mode. After a survey scan, tandem MS was performed on the most abundant precursors exhibiting a charge state from 2 to 6 of greater than  5000 intensity by isolating them in the quadrupole. HCD fragmentation was applied with 30% collision energy, and resulting fragments were detected. All mass spectra were analyzed with MaxQuant software version 1.5.5.1. MS/MS spectra were searched against the *E. coli* Uniprot protein sequence database (version July 2016) and GPM cRAP sequences (for the contaminants). Precursor mass tolerance was set to 20 ppm and 4.5 ppm for the first search where initial mass recalibration was completed and for the main search, respectively. Product ions were searched with a mass tolerance 0.5 Da. The maximal precursor ion charge state used for searching was 7. Carbamidomethylation of cysteines was searched as a fixed modification, while oxidation of methionines was searched as variable modifications. The enzyme was set to trypsin in a specific mode, and a maximum of two missed cleavages was allowed for searching. The target-decoy-based false discovery rate (FDR) filter for spectrum and protein identification was set to 1%.

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Table 1. Proteins suggested to interact with HPr in *E. coli*; K12 (Babu et al., manuscript in preparation).

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| Gene (b#) | Protein | Location | Score | Protein name | Reference |
| b2346 | MlaA | OM | 9.3 | Outer-membrane phospholipid-binding lipoprotein MlaA | ([Wu *et al.*, 2015](#_ENREF_60)) |
| b3200 | LptA | PE | 9.0 | LptA, protein essential for LPS transport across the periplasm | ([Schultz *et al.*, 2013](#_ENREF_48)) |
| b3927 | GlpF | IM | 8.9 | Glycerol uptake facilitator | ([Fu *et al.*, 2002](#_ENREF_14)) |
| b2078 | BaeS | IM | 8.8 | Sensory histidine kinase, BaeS | ([Leblanc *et al.*, 2011](#_ENREF_26)) |
| b3452 | UgpA | IM | 8.4 | Glycerol-3-phosphate ABC transporter, permease protein UgpA (TC 3.A.1.1.3) | ([Wuttge *et al.*, 2012](#_ENREF_62)) |
| b1093 | FabG | MA | 8.3 | 3-Oxoacyl-[acyl-carrier protein] reductase (EC 1.1.1.100) | ([Javidpour *et al.*, 2014](#_ENREF_21)) |
| b3462 | FtsX | IM | 7.0 | Cell division protein FtsX | ([Du *et al.*, 2016](#_ENREF_13)) |
| b3024 | YgiW | PE | 6.5 | Protein YgiW precursor involve in stress tolerance and biofilm formation | ([Lee *et al.*, 2010](#_ENREF_27); [Fukushima *et al.*, 2012](#_ENREF_15)) |
| b0474 | Adk | MA | 5.7 | Adenylate kinase | ([Ping *et al.*, 2013](#_ENREF_33)) |
| b0170 | Tsf | MA | 5.6 | Translation elongation factor EF-Ts | ([Kraal *et al.*, 1999](#_ENREF_25)) |
| b2414 | CysK | MA | 5.5 | Cysteine synthase (EC 2.5.1.47) | ([Campanini *et al.*, 2015](#_ENREF_10)) |
| b3336 | Bfr | CY | 5.4 | Bacterioferritin | ([Bradley *et al.*, 2015](#_ENREF_8)) |
| b3170 | RimP | MA | 5.4 | Bacterial 30S ribosome subunit, SSU maturation protein | ([Shajani *et al.*, 2011](#_ENREF_51)) |
| b0172 | Frr | MA | 5.3 | Ribosome recycling factor | ([Janosi *et al.*, 2000](#_ENREF_20)) |
| b2608 | RimM | MA | 5.3 | 16s rRNA processing protein, RimM | ([Shajani *et al.*, 2011](#_ENREF_51)) |
| b0678 | Nag | MA | 5.3 | Glucosamine-6P deaminase | ([Alvarez-Anorve *et al.*, 2016](#_ENREF_5)) |
| b1723 | PfkB | CY | 5.3 | Fructose-6P kinase | ([Cabrera *et al.*, 2010](#_ENREF_9)) |
| b1676 | PykF | MA | 5.3 | Pyruvate kinase | ([Al Zaid Siddiquee *et al.*, 2004](#_ENREF_2)) |
| b1200 | DhaK | CY | 5.3 | Phosphoenolpyruvate-dihydroxyacetone phosphotransferase (EC 2.7.1.121), dihydroxyacetone binding subunit DhaK | ([Gutknecht *et al.*, 2001](#_ENREF_17)) |
| b3203 | Hpf | CY | 5.3 | Ribosome hibernation promoting factor, Hpf | ([McKay and Portnoy, 2015](#_ENREF_29)) |
| b1215 | KdsA | CY | 5.3 | 2-Keto-3-deoxy-D-manno-octulosonate-8-phosphate synthase (EC 2.5.1.55) | ([Wen *et al.*, 2016](#_ENREF_57)) |
| b2417 | Crr | MA | 5.3 | PTS system, glucose-specific IIA component | ([Saier and Roseman, 1976](#_ENREF_45)) |

Locations: Cy, cytoplasm; MA, membrane associated; IM, inner membrane; OM, outer membrane; PE, periplasm

Table 2. Kinetic parameters of PykF with respect to PEP, PfkB with respect to Fru-6P, and NagB with respect to GlcN6P (in presence of 0.2 mM GlcNAc6P), all enzymes in the presence of HPr (+HPr), compared to absence of HPr (-HPr), and Adk with respect to AMP in the presence or absence of HPr-P.

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **PykF** | | **PfkB** | | **NagB** | | **Adk** | |
|  | -HPr | +HPr | -HPr | +HPr | -HPr | +HPr | -HPr-P | +HPr-P |
| Vmax, U/mg | 120 ± 5 | 117 ± 8 | 41± 3 | 40± 5 | 10±1 | 15±2 | 921 | 935 |
| h | 6.5± 0.3 | 1.8± 0.4 | 1.05± 0.30 | 0.99± 0.26 | 2.6 | 2.3 | 6.7± 2.6 | 1.3± 0.2 |
| Khalf, mM | 3.5 ± 0.1 | 0.36 ± 0.07 | 0.49±0.16 | 0.08±0.02 | 9.1±1.2 | 4.4±0.7 | 102± 6 | 750± 140 |

**U = mole/min**