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

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**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 3.5 mM to 0.36 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, allows 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.

**\body 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* ([1-4](#_ENREF_1)). 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) ([5](#_ENREF_5), [6](#_ENREF_6)). HPr energizes PTS Enzyme II complexes ([7](#_ENREF_7)) and phosphorylates the *E. coli* central regulatory protein, IIAGlc (Crr) ([8](#_ENREF_8)), as well as the PEP-dependent dihydroxyacetone kinase (DhaK) ([9](#_ENREF_9), [10](#_ENREF_10)). HPr has also been shown to interact with and regulate the *E*. *coli* glycogen phosphorylase ([11](#_ENREF_11), [12](#_ENREF_12)). 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) 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 ([13](#_ENREF_13)). The iron storage and detoxification protein, bacterioferritin (Bfr) is another interesting potential target ([14](#_ENREF_14)). HPr also appears to interact with the major cold shock protein, CspA, an RNA chaperone protein ([15](#_ENREF_15), [16](#_ENREF_16)), and the CysK subunit of cysteine synthase, an O-acetylserine sulfhydrolase ([17](#_ENREF_17)). Table 1 summarizes potential interactions of HPr with a variety of proteins.

The data presented in Table 1 suggest that HPr plays a role in ribosome-dependent protein biosynthesis ([18](#_ENREF_18)). 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 ([19](#_ENREF_19)), the anti-σ70 factor, Rsd, that under certain conditions controls translational initiation ([20](#_ENREF_20), [21](#_ENREF_21)), two ribosome processing proteins, RimM and RimP ([22](#_ENREF_22)), and Hpf, a ribosome hibernation-promoting factor ([23](#_ENREF_23)).

Kim et al. showed that one of the three pyruvate kinases in *Vibrio vulnificus,* PykA, is activated by the non-phosphorylated form of HPr ([24](#_ENREF_24)). 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 ([24](#_ENREF_24)). *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 ([25](#_ENREF_25)). The global transcriptional regulator, Cra (FruR), controls *pykF* transcription in *E. coli* ([26-29](#_ENREF_26)). Two isoforms of phosphofructokinase exist in *E. coli*, PfkA and PfkB. PfkB was shown to be inhibited by MgATP at low concentrations of Fru-6P, and this regulation is important for gluconeogenesis ([30](#_ENREF_30)).

Glucoseamine-6-phosphate deaminase (NagB) is a catabolic enzyme for the utilization of N-acetylglucosamine (GlcNAc), N-acetylmannosamine (ManNAc), N-acetylneuraminic acid (NANA) and glucosamine (GlcN). GlcNAc, ManNAc and GlcN are PTS sugars ([31](#_ENREF_31)) in E. coli so that their uptake occurs concomitantly with their phosphorylation, which produces intracellular GlcNAc-6P, ManNAc-6P and GlcN-6P, respectively. GlcNAc-6P is first deacetylated by NagA to GlcN-6P, which is then subject to deamination and isomerization by NagB, resulting in production of ammonia and fructose 6-P, the latter which enters the glycolytic pathway. The enzyme is allosterically activated by GlcNAc-6P binding, (heterotropic allosteric activation) ([32](#_ENREF_32)). GlcN-6P deaminase has two extreme structural states, with high affinity (the R state) and low affinity (the T state) for GlcN-6P. The GlcNAc-6P binding sites are present at the subunit interfaces of the hexamer, and as a consequence of GlcNAc-6P binding, the enzyme transitions to the R state. Thus, the allosteric transition activates the enzyme, increasing its apparent affinity for GlcN-6P (Km) without a change in the catalytic constant (kcat) ([33](#_ENREF_33))**.**

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 ([34](#_ENREF_34)).

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 ([24](#_ENREF_24), [35](#_ENREF_35)). Thus, the phosphorylation state of HPr can be considered as an indicator of exogenous carbon and energy availability. The co-crystallied structure of HPr with Enzyme IIA chitobiose and Enzyme I with analysis of complexes were published (PMID: 22593574, PMID: 20731394, PMID: 20080627, PMID: 19537713, PMID: 18445588). Modeling of protein-protein interactions showed

**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 lactate dehydrogenase (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. 1A). 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. 1A). 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 1 μM (Fig 1B). 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 proteomic analysis was conducted using LC-MS/MS for PykF, showing that it co-purified with HPr. By contrast, no HPr co-purified with PfkB under the same conditions (Table S1), presumably reflecting the relative affinities of these 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 pyruvate kinase (PK) and 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 ([36](#_ENREF_36), [37](#_ENREF_37)). 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. 2A; Table 2). HPr did not appear to completely antagonize inhibition by ATP (Fig 2B), 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.

***Non-Phosphorylated HPr activates GlcN-6P 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 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. The activation of NagB by GlcNAc-6P was shown in the presence and absence of HPr (Fig 3A). These kinetics were measured using 0.2 mM GlcNAc-6P in the presence and absence of 2M HPr at pH 8 and without GlcNAc-6P at pH 6.5(Fig 3B). A substantial increase in activity was observed, particularly at low concentrations of the substrate GlcN-6P at pH 6.5, without GlcNAc-6P, and at pH 8 with GlcNac-6P.

***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 4). The results revealed the inhibitory effect of the phosphorylated form of HPr on Adk (Fig 4A), 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. 4B, the inhibition of Adk is substantial, especially at low concentrations of AMP.

**Modeling of protein-protein interactions**

The docking experiments presented in Fig 6 revealed several interesting aspects of HPr interactions with PykF, PfkB, Adk, NagB.  However, first, it is important to recognize that all heteroatoms (non-amino acid atoms) were removed before submitting to docking as required by HADDOCK. The structure for PfkB, 3UMO chain A, had an ATP at position 313 before removal. The structure for Adk, 1AKE chain A, was in complex with P1,P5-bis(adenosine-5’)-pentaphosphate, an ATP analogue, and this was also removed for the docking experiments. ????

In a previous study, an interaction between HPr and glycogen phosphorylase (GlgP) was demonstrated, and the interaction between these two proteins as shown by x-ray crystallography (). This interaction involves Arg-17, Lys-24, Lys-27, Lys-40, Ser-46, Gln-51 and Lys-72. These residues appear to be important for the regulation of GlgP activity, but it is not known how they affect HPr activity.

There are two domains in PykF, the PK (pyruvate kinase catalytic domain, (residues 1-345) and the PK\_C (the C-terminal pyruvate kinase alpha/beta domain, residues 356-468) domain. In PK, there is also a disordered region of 13 amino acids (285-297). All 17 of the residues in PykF that were predicted to interact with HPr are in the PK domain, and 3 of these are in the disordered region. In HPr the range of interacting amino acyl residues are residues number 15-16, 27-32, 41-52, and 67-68 (see Table S3).

Residues in both HPr and the target enzymes, involved in the interactions with PfkB, Adk and NagB  are also presented in Table S1. According to our data present in the Table S3, HPr residues His15, 16, 17, Lys-27, 43, Ser-46, 30, 32 involved in interaction with at least 3 enzymes. Interestingly, three of the enzymes (PykF, PfkB and Adk) use His-15 as a primary site of interaction, and only NagB does not appear to interact with this Enzyme I-phosphorylatable residue.  Similarly, three of the enzymes interact with Ser-46, the residue that is phosphorylated in many bacteria that possess the HPr kinase, an enzyme that is lacking in *E. coli*. The enzymes that interact with Ser-46 of HPr include PykF, Adk and NagB, but not PfkB.

**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 of these enzymes are potential 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. 5). 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 ([38](#_ENREF_38)). We showed that the non-phosphorylated form of HPr combats ATP inhibition within the ATP concentration range of 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 GlcNAc-6P ([39-41](#_ENREF_39)). We found that HPr increases the activity of the enzyme by decreasing its apparent affinity for its substrate (Fig. 3). Thus, in all of these glycolytic enzymes, HPr affects the enzyme activity by changing the Khalf without appreciably altering the Vmax. Moreover, in all these cases, the phosphorylated form of HPr, HPr-P, was without affect, suggesting that HPr binds to these enzymes on its phosphorylatable face as is true for the interaction of HPr with the PTS enzymes, EI and the EIIA proteins ([42](#_ENREF_42)).

Adenylate kinase is an essential enzyme that catalyzes the reversible conversion of AMP and ATP to 2 molecules of ADP (Fig.5). The substrate-bound closed conformation of this enzyme is regulated by ATP (Fig. 4). Our results demonstrated substantial inhibition of adenylate kinase activity by HPr-P at low AMP concentrations. They indicate 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 exogenous PTS sugars regulates all four enzymes studied here in a coordinated fashion.

The key conserved aspects of regulation by HPr are: (1) PykF, PfkB and NagB catalyze 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 ([24](#_ENREF_24), [35](#_ENREF_35)). 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 ([31](#_ENREF_31), [43](#_ENREF_43)). Accordingly, the free HPr concentration increases. (4) HPr activates NagB, PfkB and PykF by reducing the Khalf of 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 ([44](#_ENREF_44)). (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 GlcN-6P pool. NagB redirects the flux of GlcN-6P utilization to the glycolytic pathway and is thus important and tightly regulated by two factors, GlcNAc-6P, an intermediate of the catabolic pathway and the main product of the PTS system and a PTS sensor, HPr, for the availability of GlcNAc, ManNAc, NANA and GlcN. In the allosteric regulation of NagB by HPr and GlcNAc-6P, 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 ([45](#_ENREF_45)). This was evident by the corresponding enzyme activities and the increases in the cytoplasmic concentrations of phosphoenolpyruvate, glucose-6-phosphate and 6-phosphogluconate ([45](#_ENREF_45)). 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 to monitor the cellular energy charge ([46](#_ENREF_46)). 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 mechanisms 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 ([47](#_ENREF_47)) were used for protein purification described in details in *SI Materials and methods.*

**Cloning *nagB* into pMST3** The *nagB* gene, encoding glucosamine-6-phosphate (GlcN-6P) deaminase, NagB, was PCR amplified from the *E. coli* BW25113 chromosome using oligos nagB-Bam-F : ataggatccagactgatccccctgactaccgctgaac and nagB-Sal-R ctcgtcgacttacagacctttgatattttctgcttc. See *SI Materials and methods* for full details of cloning.

**Enzyme assays.** Activities of the purified recombinant *E. coli* PykF, PfkB, NagB and Adk were routinely assayed in a cuvette at 37°C using the standard enzymatic coupling assays as described previously ([37](#_ENREF_37)).

To determine the effect of HPr on PykF, PfkB, NagB or Adk activity, 0-2.2 M HPr-P or HPr was added to an assay mixture ([25](#_ENREF_25), [49](#_ENREF_49)). 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 with the substrate, fructose-6-phosphate added to the mixture. PfkB (24 ng) was added to 100 μl of a reaction mixture containing 50mM KCl, 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, and 1.2 U of lactate dehydrogenase (LDH), and reaction rates 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 with 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.

GlcN-6P 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, 0-15 mM GlcN-6P, 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.

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. Adk (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.

**Proteomic analyses of proteins interacting with PykF and PfkB.** For the pull-down assays, PykF or PfkB in *E. coli* OE cell extracts with phosphate added to 15 mM was loaded onto Ni-NTA columns, and after washing with 1 ml of At-buffer, it was eluted with the same buffer containing 300 mM imidazole. The proteomic analyzes were conducted using LC-MS/MS with the facility at Sanford Burnham Prebys Medical Discovery Institute. *See SI Materials and methods for sample preparation.*

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Fig. 1 Allosteric activation of PykF by HPr. **A**. Steady-state kinetics of PykF were determined as a function of the PEP concentration (0.2 to 8 mM) in the absence (circles) and presence (inverted triangles) of 2 mM HPr at 0.1mM ZnSO4. The resultant kinetic parameters are presented in Table 2. **B**. The effect of varying concentrations of dephospho-HPr (triangles) on PykF activity, or HPr-P (circles) using a concentration of PEP of 1 mM. The assay is described in Materials and Methods. **C.** Activation of PykF with ZnSO4 at 1 mM HPr and 0.3 mM PEP.

Fig. 2 Allosteric activation of PfkB by HPr. **A**. Steady-state kinetics of PfkB were determined as a function of Fru-6P (0.2 to 3 mM) in the absence (squares) or presence (circles) of 2.2 mM HPr. The resultant kinetic parameters are presented in Table 2. **B**. The effect of varying concentrations of ATP on PfkB activity with dephospho-HPr (circles) or no HPr (squares) using a concentration of Fru-6P of 0.25 mM. The assay is described in Materials and Methods.

Fig. 3 Allosteric activation of NagB by HPr and GlcNAc-6P. **A**. Steady-state kinetics of NagB were determined as a function of GlcN-6P (0.2 to 20 mM) in the absence (circles) and presence (triangles) of 2 mM HPr and 0.2mM GlcNAc6P at pH 8, and in the absence (squares) and presence (rhomboids) of HPr at pH 6.5. The resultant kinetic parameters are presented in Table 2. **B**. The effect of varying concentrations of GlcNAc-6P in the absence of HPr (circles) and the presence of HPr (triangles) on NagB activity, using a concentration of 10 mM GlcN-6P at pH 8. The assay is described in Materials and Methods.

Fig. 4 **A**. ATP-dependent adenylate kinase (Adk) activity was assayed by coupling the formation of ADP to the oxidation of NADH to NAD**+** via pyruvate kinase (PYK) and lactate dehydrogenase (LDH) with continuous monitoring at 340 nm. The 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 PYK, 1.2 U of LDH and 0.2-3 mM AMP. **B.** Inhibition of adenylate kinase activity ATP + AMP ADP + ADP by HPr-P when varying the concentrations of dephospho-HPr (triangles) and phospho-HPr (circles) on Adk activity at 0.3 mM AMP.

Fig. 5 Sugar utilization pathways in *E. coli*. Bold vertical arrows highlight allosteric activation of PykF, PfkB and NagB by HPr, and inhibition of Adk by HPr-P.

**References**

1. Porcheron G, Schouler C, & Dozois CM (2016) Survival games at the dinner table: regulation of Enterobacterial virulence through nutrient sensing and acquisition. *Curr Opin Microbiol* 30:98-106.

2. Malnoy M*, et al.* (2012) Fire blight: applied genomic insights of the pathogen and host. *Annu Rev Phytopathol* 50:475-494.

3. Charkowski A*, et al.* (2012) The role of secretion systems and small molecules in soft-rot Enterobacteriaceae pathogenicity. *Annu Rev Phytopathol* 50:425-449.

4. Toth IK, Pritchard L, & Birch PR (2006) Comparative genomics reveals what makes an enterobacterial plant pathogen. *Annu Rev Phytopathol* 44:305-336.

5. Saier MH, Jr.*, et al.* (1996) Catabolite repression and inducer control in Gram-positive bacteria. *Microbiology* 142 ( Pt 2):217-230.

6. Barabote RD & Saier MH, Jr. (2005) Comparative genomic analyses of the bacterial phosphotransferase system. *Microbiol Mol Biol Rev* 69(4):608-634.

7. Reichenbach B, Breustedt DA, Stulke J, Rak B, & Gorke B (2007) Genetic dissection of specificity determinants in the interaction of HPr with enzymes II of the bacterial phosphoenolpyruvate:sugar phosphotransferase system in Escherichia coli. *J Bacteriol* 189(13):4603-4613.

8. Saier MH, Jr. & Roseman S (1976) Sugar transport. The crr mutation: its effect on repression of enzyme synthesis. *The Journal of biological chemistry* 251(21):6598-6605.

9. Gutknecht R, Beutler R, Garcia-Alles LF, Baumann U, & Erni B (2001) The dihydroxyacetone kinase of Escherichia coli utilizes a phosphoprotein instead of ATP as phosphoryl donor. *Embo J* 20(10):2480-2486.

10. Garcia-Alles LF*, et al.* (2004) Phosphoenolpyruvate- and ATP-dependent dihydroxyacetone kinases: covalent substrate-binding and kinetic mechanism. *Biochemistry* 43(41):13037-13045.

11. Seok YJ, Koo BM, Sondej M, & Peterkofsky A (2001) Regulation of E. coli glycogen phosphorylase activity by HPr. *J Mol Microbiol Biotechnol* 3(3):385-393.

12. Seok YJ*, et al.* (1997) High affinity binding and allosteric regulation of Escherichia coli glycogen phosphorylase by the histidine phosphocarrier protein, HPr. *The Journal of biological chemistry* 272(42):26511-26521.

13. Schrank TP, Wrabl JO, & Hilser VJ (2013) Conformational heterogeneity within the LID domain mediates substrate binding to Escherichia coli adenylate kinase: function follows fluctuations. *Top Curr Chem* 337:95-121.

14. Wong SG*, et al.* (2015) The B-type channel is a major route for iron entry into the ferroxidase center and central cavity of bacterioferritin. *J Biol Chem* 290(6):3732-3739.

15. Phadtare S & Severinov K (2010) RNA remodeling and gene regulation by cold shock proteins. *RNA Biol* 7(6):788-795.

16. Phadtare S & Inouye M (2008) The Cold Shock Response. *EcoSal Plus* 3(1).

17. Campanini B*, et al.* (2015) Moonlighting O-acetylserine sulfhydrylase: New functions for an old protein. *Biochim Biophys Acta* 1854(9):1184-1193.

18. Achenbach J & Nierhaus KH (2014) The mechanics of ribosomal translocation. *Biochimie*.

19. Kraal B, Lippmann C, & Kleanthous C (1999) Translational regulation by modifications of the elongation factor Tu. *Folia Microbiol (Praha)* 44(2):131-141.

20. Park YH, Lee CR, Choe M, & Seok YJ (2013) HPr antagonizes the anti-sigma70 activity of Rsd in Escherichia coli. *Proc Natl Acad Sci U S A* 110(52):21142-21147.

21. Sharma UK & Chatterji D (2010) Transcriptional switching in Escherichia coli during stress and starvation by modulation of sigma activity. *FEMS Microbiol Rev* 34(5):646-657.

22. Shajani Z, Sykes MT, & Williamson JR (2011) Assembly of bacterial ribosomes. *Annu Rev Biochem* 80:501-526.

23. Vila-Sanjurjo A (2008) Modification of the Ribosome and the Translational Machinery during Reduced Growth Due to Environmental Stress. *EcoSal Plus* 3(1).

24. Kim HM, Park YH, Yoon CK, & Seok YJ (2015) Histidine phosphocarrier protein regulates pyruvate kinase A activity in response to glucose in Vibrio vulnificus. *Mol Microbiol* 96(2):293-305.

25. Valentini G*, et al.* (2000) The allosteric regulation of pyruvate kinase. *J Biol Chem* 275(24):18145-18152.

26. Ravcheev DA*, et al.* (2014) Comparative genomics and evolution of regulons of the LacI-family transcription factors. *Front Microbiol* 5:294.

27. Saier MH, Jr. & Ramseier TM (1996) The catabolite repressor/activator (Cra) protein of enteric bacteria. *J Bacteriol* 178(12):3411-3417.

28. Ramseier TM, Bledig S, Michotey V, Feghali R, & Saier MH, Jr. (1995) The global regulatory protein FruR modulates the direction of carbon flow in Escherichia coli. *Mol Microbiol* 16(6):1157-1169.

29. Ramseier TM*, et al.* (1993) In vitro binding of the pleiotropic transcriptional regulatory protein, FruR, to the fru, pps, ace, pts and icd operons of Escherichia coli and Salmonella typhimurium. *J Mol Biol* 234(1):28-44.

30. Sabnis NA, Yang H, & Romeo T (1995) Pleiotropic regulation of central carbohydrate metabolism in Escherichia coli via the gene csrA. *J Biol Chem* 270(49):29096-29104.

31. Postma PW, Lengeler JW, & Jacobson GR (1993) Phosphoenolpyruvate:carbohydrate phosphotransferase systems of bacteria. *Microbiol Rev* 57(3):543-594.

32. Alvarez-Anorve LI*, et al.* (2011) Allosteric kinetics of the isoform 1 of human glucosamine-6-phosphate deaminase. *Biochim Biophys Acta* 1814(12):1846-1853.

33. Altamirano MM, Plumbridge JA, Horjales E, & Calcagno ML (1995) Asymmetric allosteric activation of Escherichia coli glucosamine-6-phosphate deaminase produced by replacements of Tyr 121. *Biochemistry* 34(18):6074-6082.

34. Ping J, Hao P, Li YX, & Wang JF (2013) Molecular dynamics studies on the conformational transitions of adenylate kinase: a computational evidence for the conformational selection mechanism. *Biomed Res Int* 2013:628536.

35. Deutscher J*, et al.* (2014) The bacterial phosphoenolpyruvate:carbohydrate phosphotransferase system: regulation by protein phosphorylation and phosphorylation-dependent protein-protein interactions. *Microbiol Mol Biol Rev* 78(2):231-256.

36. Cabrera R, Babul J, & Guixe V (2010) Ribokinase family evolution and the role of conserved residues at the active site of the PfkB subfamily representative, Pfk-2 from Escherichia coli. *Arch Biochem Biophys* 502(1):23-30.

37. Babul J (1978) Phosphofructokinases from Escherichia coli. Purification and characterization of the nonallosteric isozyme. *J Biol Chem* 253(12):4350-4355.

38. Torres JC, Guixe V, & Babul J (1997) A mutant phosphofructokinase produces a futile cycle during gluconeogenesis in Escherichia coli. *Biochem J* 327 ( Pt 3):675-684.

39. Alvarez-Anorve LI*, et al.* (2016) Allosteric Activation of Escherichia coli Glucosamine-6-Phosphate Deaminase (NagB) In Vivo Justified by Intracellular Amino Sugar Metabolite Concentrations. *J Bacteriol* 198(11):1610-1620.

40. 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(20):6401-6407.

41. Alvarez-Anorve LI, Calcagno ML, & Plumbridge J (2005) Why does Escherichia coli grow more slowly on glucosamine than on N-acetylglucosamine? Effects of enzyme levels and allosteric activation of GlcN6P deaminase (NagB) on growth rates. *J Bacteriol* 187(9):2974-2982.

42. Jung YS, Cai M, & Clore GM (2012) Solution structure of the IIAChitobiose-HPr complex of the N,N'-diacetylchitobiose branch of the Escherichia coli phosphotransferase system. *J Biol Chem* 287(28):23819-23829.

43. Hogema BM*, et al.* (1998) Inducer exclusion in Escherichia coli by non-PTS substrates: the role of the PEP to pyruvate ratio in determining the phosphorylation state of enzyme IIAGlc. *Mol Microbiol* 30(3):487-498.

44. Saier MH (1987) *Enzymes in metabolic pathways : a comparative study of mechanism, structure, evolution, and control* (Harper & Row, New York) pp x, 246 pages.

45. Al Zaid Siddiquee K, Arauzo-Bravo MJ, & Shimizu K (2004) Metabolic flux analysis of pykF gene knockout Escherichia coli based on 13C-labeling experiments together with measurements of enzyme activities and intracellular metabolite concentrations. *Appl Microbiol Biotechnol* 63(4):407-417.

46. Wujak M, Czarnecka J, Gorczycka M, & Hetmann A (2015) [Human adenylate kinases - classification, structure, physiological and pathological importance]. *Postepy Hig Med Dosw (Online)* 69:933-945.

47. Kitagawa M*, et al.* (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(5):291-299.

48. Rodionova IA*, et al.* (2013) Comparative genomics and functional analysis of rhamnose catabolic pathways and regulons in bacteria. *Front Microbiol* 4:407.

49. Rodionova IA*, et al.* (2012) Diversity and versatility of the Thermotoga maritima sugar kinome. *J Bacteriol* 194(20):5552-5563.

50. Wisniewski JR, Zougman A, Nagaraj N, & Mann M (2009) Universal sample preparation method for proteome analysis. *Nat Methods* 6(5):359-362.

51. Wu K*, et al.* (2015) Ultrafast Interfacial Electron and Hole Transfer from CsPbBr3 Perovskite Quantum Dots. *J Am Chem Soc* 137(40):12792-12795.

52. Schultz KM, Feix JB, & Klug CS (2013) Disruption of LptA oligomerization and affinity of the LptA-LptC interaction. *Protein Sci* 22(11):1639-1645.

53. Fu D, Libson A, & Stroud R (2002) The structure of GlpF, a glycerol conducting channel. *Novartis Found Symp* 245:51-61; discussion 61-55, 165-168.

54. Leblanc SK, Oates CW, & Raivio TL (2011) Characterization of the induction and cellular role of the BaeSR two-component envelope stress response of Escherichia coli. *J Bacteriol* 193(13):3367-3375.

55. Wuttge S*, et al.* (2012) Determinants of substrate specificity and biochemical properties of the sn-glycerol-3-phosphate ATP binding cassette transporter (UgpB-AEC2 ) of Escherichia coli. *Mol Microbiol* 86(4):908-920.

56. Javidpour P*, et al.* (2014) Biochemical and structural studies of NADH-dependent FabG used to increase the bacterial production of fatty acids under anaerobic conditions. *Appl Environ Microbiol* 80(2):497-505.

57. Du S, Pichoff S, & Lutkenhaus J (2016) FtsEX acts on FtsA to regulate divisome assembly and activity. *Proc Natl Acad Sci U S A* 113(34):E5052-5061.

58. Fukushima K, Kumar SD, & Suzuki S (2012) YgiW homologous gene from Pseudomonas aeruginosa 25W is responsible for tributyltin resistance. *J Gen Appl Microbiol* 58(4):283-289.

59. Lee J, Hiibel SR, Reardon KF, & Wood TK (2010) Identification of stress-related proteins in Escherichia coli using the pollutant cis-dichloroethylene. *J Appl Microbiol* 108(6):2088-2102.

60. Kraal B, Lippmann C, & Kleanthous C (1999) Translational regulation by modifications of the elongation factor Tu. *Folia Microbiol (Praha)* 44(2):131-141.

61. Bradley JM*, et al.* (2015) Three Aromatic Residues are Required for Electron Transfer during Iron Mineralization in Bacterioferritin. *Angew Chem Weinheim Bergstr Ger* 127(49):14976-14980.

62. Janosi L*, et al.* (2000) Mutations influencing the frr gene coding for ribosome recycling factor (RRF). *J Mol Biol* 295(4):815-829.

63. Gutknecht R, Beutler R, Garcia-Alles LF, Baumann U, & Erni B (2001) The dihydroxyacetone kinase of Escherichia coli utilizes a phosphoprotein instead of ATP as phosphoryl donor. *Embo J* 20(10):2480-2486.

64. McKay SL & Portnoy DA (2015) Ribosome hibernation facilitates tolerance of stationary-phase bacteria to aminoglycosides. *Antimicrob Agents Chemother* 59(11):6992-6999.

65. Wen L, Zheng Y, Li T, & Wang PG (2016) Enzymatic synthesis of 3-deoxy-d-manno-octulosonic acid (KDO) and its application for LPS assembly. *Bioorg Med Chem Lett* 26(12):2825-2828.

66. Saier MH, Jr. & Roseman S (1976) Sugar transport. The crr mutation: its effect on repression of enzyme synthesis. *J Biol Chem* 251(21):6598-6605.

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 | ([51](#_ENREF_51)) |
| b3200 | LptA | PE | 9.0 | LptA, protein essential for LPS transport across the periplasm | ([52](#_ENREF_52)) |
| b3927 | GlpF | IM | 8.9 | Glycerol uptake facilitator | ([53](#_ENREF_53)) |
| b2078 | BaeS | IM | 8.8 | Sensory histidine kinase, BaeS | ([54](#_ENREF_54)) |
| b3452 | UgpA | IM | 8.4 | Glycerol-3-phosphate ABC transporter, permease protein UgpA (TC 3.A.1.1.3) | ([55](#_ENREF_55)) |
| b1093 | FabG | MA | 8.3 | 3-Oxoacyl-[acyl-carrier protein] reductase (EC 1.1.1.100) | ([56](#_ENREF_56)) |
| b3462 | FtsX | IM | 7.0 | Cell division protein FtsX | ([57](#_ENREF_57)) |
| b3024 | YgiW | PE | 6.5 | Protein YgiW precursor involve in stress tolerance and biofilm formation | ([58](#_ENREF_58), [59](#_ENREF_59)) |
| b0474 | Adk | MA | 5.7 | Adenylate kinase | ([34](#_ENREF_34)) |
| b0170 | Tsf | MA | 5.6 | Translation elongation factor EF-Ts | ([60](#_ENREF_60)) |
| b2414 | CysK | MA | 5.5 | Cysteine synthase (EC 2.5.1.47) | ([17](#_ENREF_17)) |
| b3336 | Bfr | CY | 5.4 | Bacterioferritin | ([61](#_ENREF_61)) |
| b3170 | RimP | MA | 5.4 | Bacterial 30S ribosome subunit, SSU maturation protein | ([22](#_ENREF_22)) |
| b0172 | Frr | MA | 5.3 | Ribosome recycling factor | ([62](#_ENREF_62)) |
| b2608 | RimM | MA | 5.3 | 16s rRNA processing protein, RimM | ([22](#_ENREF_22)) |
| b0678 | NagB | MA | 5.3 | Glucosamine-6P deaminase | ([39](#_ENREF_39)) |
| b1723 | PfkB | CY | 5.3 | Fructose-6P kinase | ([36](#_ENREF_36)) |
| b1676 | PykF | MA | 5.3 | Pyruvate kinase | ([45](#_ENREF_45)) |
| b1200 | DhaK | CY | 5.3 | Phosphoenolpyruvate-dihydroxyacetone phosphotransferase (EC 2.7.1.121), dihydroxyacetone binding subunit DhaK | ([63](#_ENREF_63)) |
| b3203 | Hpf | CY | 5.3 | Ribosome hibernation promoting factor, Hpf | ([64](#_ENREF_64)) |
| b1215 | KdsA | CY | 5.3 | 2-Keto-3-deoxy-D-manno-octulosonate-8-phosphate synthase (EC 2.5.1.55) | ([65](#_ENREF_65)) |
| b2417 | Crr | MA | 5.3 | PTS system, glucose-specific IIA component | ([66](#_ENREF_66)) |

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

Table 2. Kinetic parameters of (1) - PykF with respect to PEP, (2) - PfkB with respect to Fru-6P, (3) - NagB with respect to GlcN-6P (in the presence of 0.2 mM GlcNAc-6P) and Adk with respect to AMP. All enzymes were assayed in the presence of HPr (+HPr) or phosphorylated HPr (+HPr-P) compared to absence of HPr (-HPr).

|  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
|  | **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 | 736±24 | 936±93 |
| 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**