The phosphocarrier protein HPr of the bacterial phosphotransferase system globally regulates energy metabolism by directly interacting with multiple enzymes in *Escherichia coli*

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The histidine-phosphorylatable phosphocarrier protein (HPr) is an essential component of the sugar-transporting phosphotransferase system (PTS) in many bacteria. Recent interactome findings suggested that HPr interacts with several carbohydrate-metabolizing enzymes, but whether HPr plays a regulatory role was unclear. Here, we provide evidence that HPr 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 is either phosphorylated on a histidyl residue (HPr-P) or non-phosphorylated (HPr). PykF is activated only by non-phosphorylated HPr, which decreases the PykF K_{half} for phosphoenolpyruvate by 10-fold (from 3.5 to 0.36 mM), thus influencing glycolysis. PfkB activation by HPr, but not by HPr-P, resulted from a decrease in the K_{half} for fructose-6-P, which likely influences both gluconeogenesis and glycolysis. Moreover, NagB activation by HPr was important for the utilization of amino sugars, and allosteric inhibition of Adk activity by HPr-P, but not by HPr, allows HPr to regulate the cellular energy charge coordinately with glycolysis. These observations suggest that HPr serves as a directly interacting global regulator of carbon and energy metabolism and probably of other physiological processes in enteric bacteria.

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* species (1–4). In these organisms and many others, the histidine phosphorylatable phosphocarrier protein (HPr)[2] is an essential constituent of the sugar-transporting phosphoenolpyruvate (PEP):sugar phosphotransferase system (PTS) (5, 6). HPr energizes PTS Enzyme II complexes (7–10) and phosphorylates the *E. coli* central regulatory protein, IIA^{Glc} (Crr) (10). HPr has also been shown to interact with and regulate the *E. coli* glycogen phosphorylase (11–13), and recently, the mannitol utilization regulator MtlR (13). However, interactions of HPr with other cellular constituents for purposes of regulation have not been demonstrated, and thus, HPr is not recognized as a global regulator.

Recently obtained *E. coli* interactome data[3] have suggested that other carbohydrate metabolic enzymes interact with HPr (supplemental Table S1). 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 (14). The iron storage and detoxification protein, bacterioferritin, is another interesting potential target. HPr also appears to interact with the major cold shock protein, CspA, an RNA chaperone protein, and the CysK subunit of cysteine synthase, an *O*-acetylserine sulfhydrolase. supplemental Table S1 summarizes potential interactions of HPr with a variety of proteins.

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This article contains supplemental "Experimental procedures" and Tables S1 and S2.

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[2] The abbreviations used are: HPr, histidine-phosphorylatable phosphocarrier protein; PTS, phosphotransferase system; LDH, lactate dehydrogenase; Adk, adenylate kinase; PfkB, phosphofructokinase; NagB, glucosamine-6-phosphate deaminase; K_{half}, the concentration of substrate that produces a half-maximal enzyme velocity; PEP, phosphoenolpyruvate; PK, pyruvate kinase; EI, Enzyme I; PykF, pyruvate kinase F; KdsA, 3-deoxy-D-manno-octulosonate-8-phosphate synthase; Pgi, phosphoglucose isomerase; Zwf, glucose-6-phosphate dehydrogenase.

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The data presented in supplemental Table S1 suggest that HPr plays a role in ribosome-dependent protein biosynthesis. 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, the anti-σ^{70} factor, Rsd, that under certain conditions controls translational initiation, two ribosome processing proteins, RimM and RimP, and Hpf, a ribosome hibernation-promoting factor.

Kim *et al.* (15) showed that one of the three pyruvate kinases in *Vibrio vulnificus*, PykA, is activated by the non-phosphorylated form of HPr. The PTS, which catalyzes 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.* (15) examined the *E. coli* PykA, but regulation could not be demonstrated. *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 reflects the central position of PykF in cellular metabolism (16). The global transcriptional regulator, Cra (FruR), controls *pykF* transcription in *E. coli* (17–20). Two isoforms of phosphofructokinase exist in *E. coli*, PfkA and PfkB. PfkB was shown to be inhibited by MgATP at low concentrations of Fru-6-P, and this regulation is important for gluconeogenesis (21).

Glucosamine-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 in *E. coli* so that their uptake occurs concomitantly with their phosphorylation, producing intracellular GlcNAc-6-P, ManNAc-6-P, and GlcN-6-P, respectively (22). GlcNAc-6-P is first deacetylated by NagA to GlcN-6-P, which is then subject to deamination and isomerization by NagB, resulting in the production of ammonia and fructose-6-P; the latter enters the glycolytic pathway. The enzyme is allosterically activated by GlcNAc-6-P binding (heterotrophic activation) (23). GlcN-6-P deaminase has two extreme structural states, with high affinity (the R state) and low affinity (the T state) for GlcN-6-P. The GlcNAc-6-P-binding sites are present at the subunit interfaces of the hexamer, and as a consequence of GlcNAc-6-P binding, the enzyme transitions to the R state. Thus, the allosteric transition activates the enzyme, increasing its apparent affinity for GlcN-6-P (K_m) without changing the catalytic constant (k_{cat}) (24).

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 three 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 (25).

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, whereas 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, *N*-acetylglucosamine, or fructose is present in the extracellular medium, and in the phosphorylated form in the absence of a PTS sugar (15, 26). Thus, the phosphorylation state of HPr can be considered as an indicator of exogenous carbon and energy availability. The co-crystallized structures of HPr with the chitobiose Enzyme IIA and with Enzyme I, with analysis of the complexes, have been published (27–31). Modeling of protein-protein interactions for PykF, PfkB, and Adk as reported here suggests that HPr binding involves residues His-15, Thr-16, Lys-27, Lys-49, Gln-51, and Thr-52, and similar interactions had been demonstrated previously for the glucose EIIA/HPr phosphoryl transfer complex. Interestingly, modeling of the NagB/HPr interaction suggested that this complex exhibits very different characteristics.

Results

Non-phosphorylated HPr activates pyruvate kinase, PykF

E. coli PykF activity was measured using a coupled assay involving lactate dehydrogenase (LDH) (see "Experimental procedures"). 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 2 μM HPr (Fig. 1*A*). Parameters derived from the kinetics are presented in Table 1. The presence of HPr caused the K_{half} for PEP to decrease 10-fold. Thus, HPr primarily influenced the affinity of PykF for PEP and accordingly, activated PykF at low PEP concentrations (Fig. 1*A*). The Hill coefficient was also reduced from 6.5 to 1.8.

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 with HPr. The phosphorylated form was obtained by preincubation with PEP and EI for 30 min at 37 °C. HPr-P had no effect on PykF activity, but HPr showed a positive effect, with a measured K_d for HPr of 1 μM (Fig. 1*B*). The best effect observed with HPr at a concentration of 1 μM depended on the presence of 100 μM $ZnSO_4$ (K_a = 74 μM). Titration with zinc at an HPr concentration of 1 μM is presented in Fig. 1*C*. Zinc had only an inhibitory effect when HPr was absent.

A proteomic analysis was conducted using LC-MS/MS for PykF, giving an intensity score of 13×10^6, suggesting that it co-purified with HPr. By contrast, no HPr could be shown to co-purify with PfkB under the same conditions, presumably reflecting the relative affinities of these two enzymes for HPr.

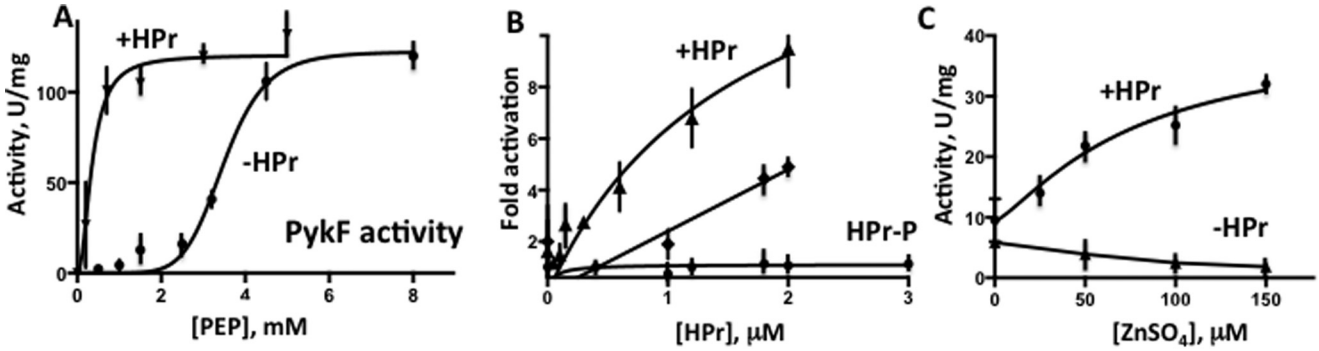

Figure 1. Allosteric activation of PykF by HPr. *A,* steady-state kinetics of PykF were determined as a function of the PEP concentration (0 to 8 mM) in the absence (*circles*) or presence (*inverted triangles*) of 2 μM HPr, all at 0.1 mM $ZnSO_4$. The resultant kinetic parameters are presented in Table 1. *B,* the effect of varying concentrations of dephospho-HPr (*triangles*) (*diamonds* in the absence of $ZnSO_4$) or HPr-P (*circles*) on PykF activity using 1 mM PEP. The assay is described under "Experimental procedures." *C,* activation of PykF with varying concentrations of $ZnSO_4$ at 0.3 mM PEP and with (*circles*) or without (*triangles*) 1 μM HPr. Units are defined as μmol/min.

Table 1

Kinetic parameters of: 1) PykF with respect to PEP; 2) PfkB with respect to Fru-6-P; 3) NagB with respect to GlcN-6-P (in the presence of 0.2 mM GlcNAc-6-P at pH 8) and Adk with respect to AMP

All enzymes were assayed in the absence (−HPr) or presence (+HPr) of HPr or absence (−HPr-P) or presence (+HPr-P) of the phosphorylated HPr. K_{half} is the concentration of substrate that produces a half-maximal enzyme velocity (V_{max}, maximum velocity).

	PykF		PfkB		NagB		Adk	
	−HPr	+HPr	−HPr	+HPr	−HPr	+HPr	−HPr-P	+HPr-P
V_{max} (U/mg)[a]	120 ± 5	117 ± 8	41 ± 3	40 ± 5	10 ± 1	15 ± 2	736 ± 24	936 ± 93
Hill coefficient	6.5 ± 0.3	1.8 ± 0.4	1.05 ± 0.30	0.99 ± 0.26	2.6 ± 0.7	2.3 ± 0.5	6.7 ± 2.6	1.3 ± 0.2
K_{half} (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	0.1 ± 0.01	0.75 ± 0.14

[a] U = μmol/min.

Non-phosphorylated HPr activates phosphofructokinase, PfkB

E. coli PfkB activity was measured using the coupled assay described under "Experimental procedures." PfkB activity was previously known to be allosterically inhibited by ATP in the presence of 50 mM KCl (32, 33). To evaluate the effect of free HPr, steady-state kinetics were measured in the presence and absence of 2.2 μM HPr. Although HPr showed no effect on the V_{max} or the Hill coefficient (h), the presence of HPr led to a 6-fold decrease in the K_{half} for Fru-6-P. This result implies that HPr regulates PfkB activity by increasing the affinity of PfkB for its substrate, Fru-6-P. It is important to emphasize that in the presence of 50 mM KCl and 1 mM ATP, the non-phosphorylated form of HPr activated PfkB, decreasing the K_{half} for Fru-6-P from 0.49 to 0.08 mM (Fig. 2*A*; Table 1). HPr did not appear to completely antagonize inhibition by ATP (Fig. 2*B*), but KCl strengthened the inhibitory 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 whether HPr-P affected PfkB activity, EI (10 μg/ml), PEP (1 mM), and different concentrations of HPr-P were added in the PfkB assay reaction. The addition of HPr-P, when fully phosphorylated, up to 3 μM, did not result in activation or inhibition, although HPr saturates PfkB for activation at about 2.2 μM.

Non-phosphorylated HPr activates GlcN-6-P deaminase, NagB

E. coli NagB activity was measured as described under "Experimental procedures." This assay is based on the conversion of GlcN-6-P to fructose 6-phosphate by NagB, followed by isomerization to glucose 6-phosphate by Pgi and oxidation of glucose 6-phosphate to gluconate 6-phosphate by Zwf. The activation of NagB by GlcNAc-6-P and by HPr was shown by assaying the enzyme in the presence and absence of HPr (Fig. 3*A*). The kinetics were measured using 0.2 mM GlcNAc-6-P in the presence and absence of 2 μM HPr at pH 8 and without GlcNAc-6-P at pH 6.5 (Fig. 3*A*, Table 1). A substantial increase in activity was observed, particularly at low concentrations of the substrate GlcN-6-P at pH 6.5 without GlcNAc-6-P, and at pH 8 with GlcNAc-6-P.

Phosphorylated HPr inhibits Adk

The activity of Adk was measured in a pyruvate kinase (PK) and LDH-coupled assay as described under "Experimental procedures." The kinetics were measured in the presence and absence of 1 μM HPr-P or HPr (Fig. 4). The results revealed the inhibitory effect of the phosphorylated form of HPr on Adk (Fig. 4*A*), although HPr itself was without effect.

Substantial inhibition was observed at concentrations of AMP less than 1 mM. The titration of HPr-P as shown in Fig. 4*B*, revealed that saturation occurred at a concentration of 1.0 μM; the K_i for HPr-P binding was 0.1 μM. Table 1 presents the changes in the kinetic parameters upon HPr-P binding. The K_{half} increased more than 7-fold, and as shown in Fig. 4*A*, the inhibition of Adk is substantial, especially at low concentrations of AMP.

Structural implications of protein-protein interactions

An interaction between HPr and glycogen phosphorylase (GlgP) had been shown previously (11, 12). This interaction involves HPr residues 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. There are two domains in PykF,

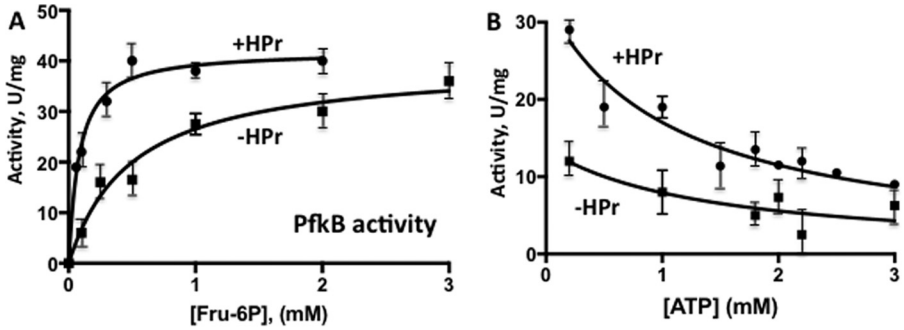

Figure 2. Allosteric activation of PfkB by HPr. *A,* steady-state kinetics of PfkB activity were determined as a function of Fru-6-P (0 to 3 mM) in the absence (*squares*) or presence (*circles*) of 2.2 μM HPr. The resultant kinetic parameters are presented in Table 1. *B,* the effect of varying concentrations of ATP on PfkB activity with dephospho-HPr (*circles*) or no HPr (*squares*) using a concentration of Fru-6-P of 0.25 mM. The assay is described under "Experimental procedures." Units are defined as μmol/min.

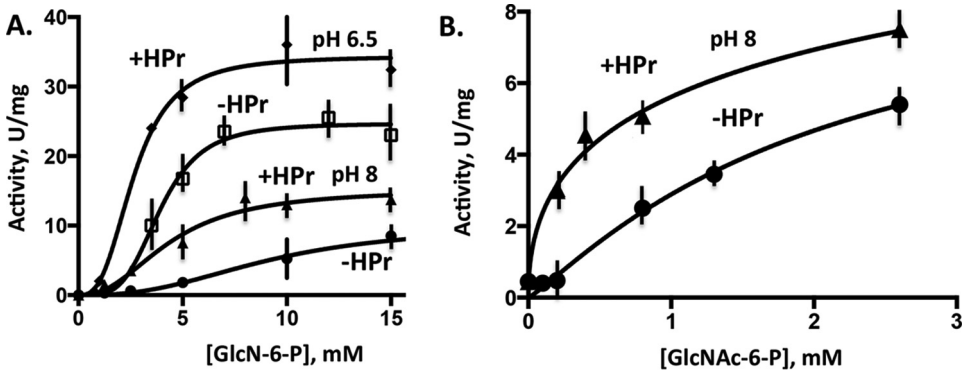

Figure 3. Allosteric activation of NagB by HPr and GlcNAc-6-P. *A,* steady-state kinetics of NagB were determined as a function of GlcN-6-P (0 to 15 mM) at 0.2 mM GlcNAc-6-P in the absence (*circles*) and presence (*triangles*) of 2 μM HPr at pH 8, and in the absence (*open squares*) and presence (*diamonds*) of HPr at pH 6.5. The resultant kinetic parameters are presented in Table 1. *B,* the effect of varying concentrations of GlcNAc-6-P in the absence of HPr (*circles*) and the presence of HPr (*triangles*) on NagB activity, using a concentration of 10 mM GlcN-6-P at pH 8. The assay is described under "Experimental procedures." Units are defined as μmol/min.

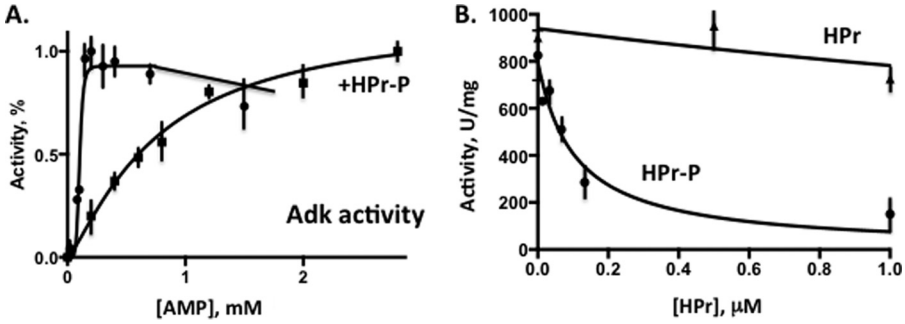

Figure 4. Inhibition of ATP-dependent Adk by phospho-HPr. *A,* Adk activity was assayed by coupling the formation of ADP to the oxidation of NADH to NAD^+ via pyruvate kinase (*PYK*) and lactate dehydrogenase (*LDH*) with continuous monitoring at 340 nm. The reaction mixture containing 50 mM Tris-HCl buffer (pH 7.5), 10 mM $MgSO_4$, 1.2 mM ATP, 1.2 mM PEP, 0.3 mM NADH, 1.2 units of PYK, 1.2 units of LDH, and 0–3 mM AMP with (*squares*) or without (*circles*) HPr-P (1 μM). *B,* inhibition of adenylate kinase activity by HPr-P when varying the concentrations of dephospho-HPr (*triangles*) or phospho-HPr (*circles*) on Adk activity at 0.3 mM AMP. Units are defined as μmol/min.

the PK (pyruvate kinase catalytic domain, residues 1–345), and PK_C (the C-terminal pyruvate kinase α/β domain, residues 356–468). In PK, there is a disordered region of 13 amino acids (residues 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 17 residues were in the disordered region. The presence of zinc was shown to improve binding of HPr to PykF, suggesting that residues 285–297 form the allosteric binding site for zinc. In HPr, the range of interacting aminoacyl residues are residue numbers 15–16, 27–32, 41–52, and 67–68 (see supplemental Table S2).

Residues in both HPr and the target enzymes, predicted to be involved in the interactions with PfkB, Adk, and NagB, are also presented in supplemental Table S2; models of these interactions are shown in Fig. 5. According to the data presented in supplemental Table S2, HPr residues His-15, Thr-16, Lys-17, Lys-27, Glu-43, and Ser-46 are involved in interactions with at least 3 enzymes (PykF, PfkB, and Adk). Interestingly, these three enzymes use His-15 as a primary site of interaction, whereas only NagB does not appear to interact with this Enzyme I-phosphorylatable residue. The same three enzymes (PykF, PfkB, and Adk) interact with HPr Ser-46, the residue that

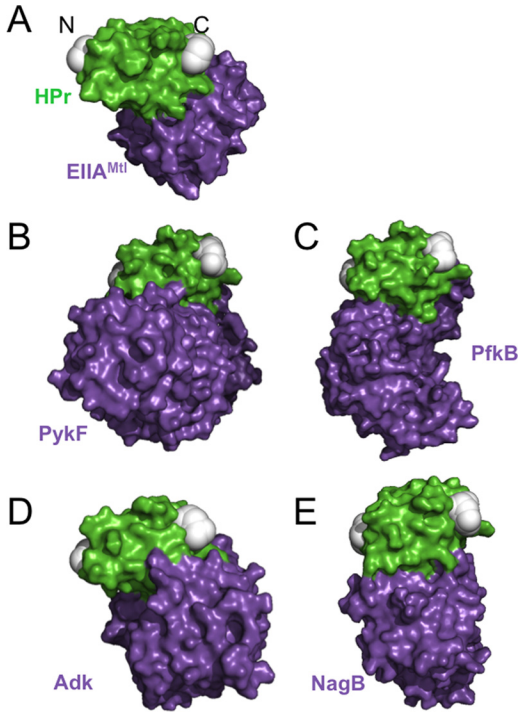

Figure 5. Protein-protein interactions (*PPI*) between HPr and metabolic proteins. *A,* the PPI of HPr with the mannitol EIIA protein ($EIIA^{Mtl}$) had been known before this study was conducted. *B–E,* the interactions between HPr and PykF, PfkB, Adk, and NagB, respectively, were identified in a recent AP/MS study.[3] We also confirmed the HPr–PfkB interaction by a bacterial two-hybrid assay. Docking models of HPr were oriented the same way in each model, showing that their interaction partners bind to overlapping sites. *N* and *C* (*white spheres*) indicate the N and C termini of HPr. Note that binding changes the conformation of HPr so that each model looks slightly different. The Protein Data Bank codes and chains used for HPr, PykF, PfkB, NagB, and Adk were 3CCD:A, 4YNG:A, 3UMO:A, 1AKE:A, and 1FS5:A, respectively. Protein Data Bank code 1J6T was used for HPr + $EIIA^{Mtl}$.

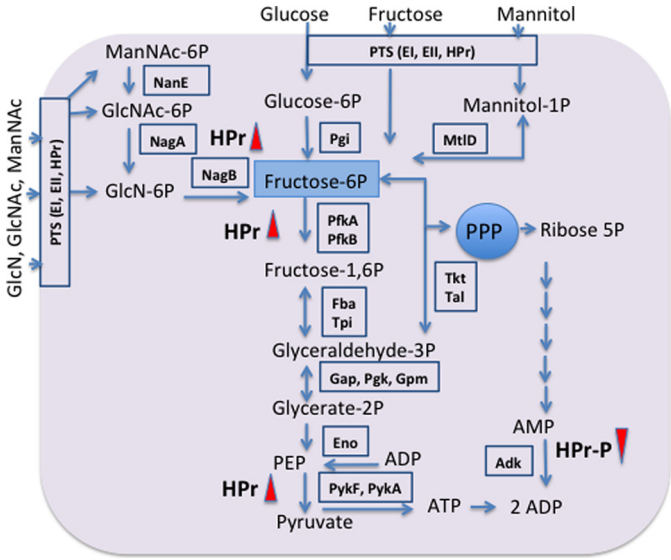

Figure 6. Sugar utilization pathways in *E. coli*. *Vertical arrows* adjacent to HPr or HPr-P highlight allosteric activation of PykF, PfkB, and NagB by HPr, and inhibition of Adk by HPr-P, respectively.

is phosphorylated in many bacteria that possess the HPr kinase (34), an enzyme that is lacking in *E. coli*. Surprisingly, NagB does not appear to interact with the key residues, His-15 and Ser-46, in HPr.

Discussion

E. coli HPr interactome analyses (supplemental Table S1) suggested direct interactions with the glycolytic enzymes, PykF and PfkB, and a key enzyme for amino sugar catabolism that feeds into glycolysis, NagB, as well as adenylate kinase. All of these enzymes were considered to be 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. 6). The data presented reveal activation of PykF by the non-phosphorylated form of HPr with a decrease in the K_{half} of around 10-fold. The HPr affinity is apparently enhanced by zinc (Fig. 1*B*), suggesting that zinc improves binding of HPr to PykF for activation.

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 in addition to glycolysis (35). We showed that the non-phosphorylated form of HPr combats ATP inhibition within the ATP concentration range of 0.1 to 3 mM (Fig. 2). The flux of intracellular glucose-6-P 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-6-P (36–38). We found that HPr increases the activity of the enzyme by increasing its apparent affinity for its substrate (Fig. 3). Thus, in all of these glycolytic enzymes, HPr affects the enzyme activity by changing the K_{half} without appreciably altering the V_{max}. Moreover, in the cases of PykF and PfkB, 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 (27).

Adenylate kinase is an essential enzyme that catalyzes the reversible conversion of AMP and ATP to 2 molecules of ADP (Fig. 6). The substrate-bound closed conformation of this enzyme is regulated by AMP (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 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. 2) These enzymes are all activated by HPr. 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 (15, 26). 3) Such sugars, when present, give rise to the dephospho form of the protein due to sugar phosphorylation, but when exogenous PTS sugars are absent, HPr-P should pre-

dominate. 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 (22, 39). 4) Accordingly, the free HPr concentration increases. HPr activates NagB, PfkB, and PykF by reducing the K_{half} of these enzymes for their substrates, and the overall glycolytic flux should therefore increase with availability of an exogenous PTS sugar. 5) All three glycolytic enzymes are known to be rate-limiting (40). The biosynthetic pathway producing UDP-GlcNAc for incorporation into cell wall components involves *glmS*, *glmM*, and *glmU* gene products and utilizes the cytoplasmic GlcN-6-P pool. NagB redirects the flux of GlcN-6-P utilization to the glycolytic pathway and is thus important and tightly regulated by two factors, GlcNAc-6-P, an intermediate of the catabolic pathway and the main product of the PTS, and a PTS sensor, HPr, for the availability of GlcNAc, ManNAc, and GlcN. In the allosteric regulation of NagB by HPr and GlcNAc-6-P, catabolism of amino sugars is effectively coordinated with other metabolic reactions occurring in the *E. coli* cell.

In a PykF$^-$ mutant, flux through the glycolytic pathway was reduced, whereas that through the oxidative pentose phosphate pathway was increased (41). This was evident by the corresponding enzyme activities and the increases in the cytoplasmic concentrations of phosphoenolpyruvate, glucose 6-phosphate, and 6-phosphogluconate (41). 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 activated PykF. Thus, activation of PykF by HPr should lead to global changes in *E. coli* carbon and energy metabolism.

Adenylate kinase allows the reversible 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 these nucleotides and to monitor the cellular energy charge (42). The kinetics of adenylate kinase crucially changes in the presence of HPr-P, in response 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.

Experimental procedures

Protein purification

Recombinant proteins containing an N-terminal His_6 tag were overexpressed in *E. coli* and purified using Ni^{2+}-chelating chromatography. The *E. coli* OE strains, overexpressing PykF, PfkB, Adk, PtsH, or PtsI from the ASKA collection (43) and the NagB strain (*nagB* cloned in pMST3, transferred into *E. coli* BL21(DE3)), were used for protein purification using Ni-NTA-agarose minicolumn with At buffer containing 50 mM Tris-HCl buffer (pH 8), 0.5 mM NaCl, 5 mM imidazole, and 0.03 or 0.3 % (for PtsH) Tween 20 described in detail under supplemental "Experimental procedures".

Cloning nagB into pMST3

The *nagB* gene, encoding glucosamine-6-phosphate (GlcN-6-P) deaminase, NagB, was PCR amplified from the *E. coli* BW25113 chromosome using oligos nagB-Bam-F, ataggatccagactgatccccctgactaccgctgaac and nagB-Sal-R, ctcgtcgacttacagacctttgatattttctgcttc. See supplemental "Experimental procedures" for full details of the 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 standard enzymatic coupling assays following the decrease in absorbance at 340 nm resulting from the oxidation of NADH as described previously (33).

To determine the effect of HPr on PykF, PfkB, NagB, or Adk activity, 0–2.2 μM HPr-P or HPr was added to an assay mixture (16, 44). HPr was phosphorylated in the assay mixture containing 100 mM Tris (pH 8), 2 mM DTT, 8 mM PEP, 10 mM $MgCl_2$ and EI and incubated for 40 min at 30 °C. Subsequently, up to 3 μM HPr-P, 10 μg/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 with those for the two sets of control samples: one control without the tested enzyme and another without AMP. The K_{half} and relative V_{max} values were determined using GraphPad Prism software.

PfkB-dependent fructose-6-P (Fru-6-P) phosphorylation converts ATP to ADP, and ADP is used by 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 (24 ng) was added to 100 μl of a reaction mixture containing 50 mM KCl, 50 mM Tris-HCl buffer (pH 7.5), 10 mM $MgSO_4$, 1.2 mM ATP, 1.2 mM PEP, 0.3 mM NADH, 1.2 units of PK, and 1.2 units of LDH, and reaction rates were compared with controls in which fructose 6-phosphate was absent.

Pyruvate kinase (PykF) activity was measured 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 $MgSO_4$, 1.5 mM ADP, 0–8 mM PEP, 0.3 mM NADH, 0.2 M KCl, 100 μM $ZnSO_4$, 5 mM phosphate, and 1.2 units of LDH. Titration with HPr was similarly assayed, but for measuring the effect of HPr-P on PykF activity, 2 mM DTT, 2 mM PEP, and 10 μg/ml of EI were also added to the mixture.

GlcN-6-P deaminase activity was assayed by coupling the formation of fructose 6-phosphate with the phosphoglucose isomerase (Pgi) and glucose-6-phosphate dehydrogenase (Zwf)-dependent reduction of NADP. NagB (20 ng) was added to 100 μl of a reaction mixture containing 100 mM Tris-HCl buffer (pH 8 or 6.5), 5 mM $MgSO_4$, 0–15 mM GlcN-6-P, 2 mM NADP, 1.2 units of Pgi, 1.2 units of Zwf, and 5 mM phosphate. To determine the effect of HPr on NagB activity, 0–2 μM 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 with 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. The PEP and the ADP produced react to generate pyruvate and ATP, and the pyruvate formed is reduced to lactate. Adk (1 ng) was added to 100 μl of a kinase reaction mixture containing 50 mM Tris-HCl buffer (pH 7.5), 10 mM $MgSO_4$, 1.2 mM ATP, 1.2 mM PEP, 0.3 mM NADH, 1.2 units of PK, 1.2 units of LDH, 2 mM 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 *Escherichia coli* OE cell extracts with phosphate added to 15 mM was loaded onto nickel-nitrilotriacetic acid 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 supplemental "Experimental procedures" for sample preparation.

Author contributions—I. R. and M. S. designed the study, analyzed the data, and wrote the paper. Z. Z. assisted with the nagB gene cloning. A. E., M. B., J. M., M. S., and P. U. provided protein–protein interaction analyses. N. G. and P. U. performed protein–protein interactions modeling. All authors reviewed the results and approved the final version of the manuscript.

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