# Investigation of transphosphorylation between chemotaxis proteins and the phosphoenolpyruvate:sugar phosphotransferase system

Mark S. Johnson, Edward H. Rowsell**, Barry L. Taylor*

*Department of Microbiology and Molecular Genetics, Loma Linda University, Loma Linda, CA 92350, USA*

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**Abstract Transphosphorylation between the chemotaxis proteins and phosphoenolpyruvate:sugar phosphotransferase system (PTS) from *Escherichia coli* was investigated by incubating the CheA, CheW and CheY proteins of the chemotaxis cascade, and Enzyme I, HPr and Enzyme II$^{mtl}$ of the PTS with [$\gamma$-$^{32}$P]ATP or [$^{32}$P]phosphoenolpyruvate in the presence and absence of cell extract. In the absence of cell extract, ATP phosphorylated CheA, but in the presence of cell extract, Enzyme I was also phosphorylated. Phosphoenolpyruvate phosphorylated only PTS components. The transphosphorylation of Enzyme I by ATP did not require chemotaxis proteins, and likely occurred through acetate kinase. Regardless of phosphorylation state, the HPr protein did not inhibit the rate of ATP-dependent phosphorylation of the CheA or the CheY protein. It is concluded that chemotaxis to PTS substrates is not mediated by transphosphorylation between the PTS and chemotaxis systems.**

*Key words:* Bacterial chemotaxis; Phosphotransferase system; Enzyme I; CheA protein; CheY protein; Acetate kinase; *Escherichia coli*

## 1. Introduction

Chemotaxis to sugars that are transported into the cell by the phosphoenolpyruvate:sugar phosphotransferase system (PTS) in *Escherichia coli* and *Salmonella typhimurium* requires two parallel transphosphorylation cascades – one pathway energized by ATP and the other pathway energized by phosphoenolpyruvate (PEP) [1,2]. The ATP-dependent cascade is common to all chemotaxis pathways and communicates information about changes in the external environment to a switch on the flagellar motor [1,3]. Most non-PTS chemoeffectors are detected by one of four receptors that are integral membrane proteins known as methyl accepting chemotaxis proteins (MCPs). The cytoplasmic domain of the MCP associates with the chemotaxis proteins, CheA and CheW, so that conformational changes initiated by a chemoeffector binding to the periplasmic domain of the MCP can modulate ATP-dependent autophosphorylation of CheA in the cytoplasm [3]. The phosphoryl group on CheA is transferred to the CheY protein and phospho-CheY binds to the flagellar motor switch, inducing a change from counterclockwise rotation (smooth swimming behavior) to clockwise rotation (tumbling) of the flagella. Adaptation to an attractant occurs when signaling by activated MCPs is attenuated by methylesterification of specific glutamyl residues on the cytoplasmic domain of the MCP.

Chemotaxis to PTS sugars, in contrast to MCP-mediated chemotaxis, requires transport of the sugar via a PEP-energized cascade [2]. Adaptation in PTS chemotaxis does not require methylation of an MCP [4]. Enzyme I and the HPr protein transfer a phosphoryl group from PEP to a sugar-specific Enzyme II that concomitantly phosphorylates the sugar and transports it into the cytoplasm [2]. As the concentration of external sugar changes, the rate of sugar transport also changes. The change in rate of transport apparently alters the level of CheY phosphorylation by an unknown mechanism that requires the CheA and CheW proteins [1]. Evidence has been reported that suggests that the HPr protein interacts with the chemotaxis cascade [5,6]. Slow transphosphorylation has been observed from phospho-CheA to the NtrC, OmpR, and SpoOA proteins (regulators of the nitrogen, osmolarity, and sporulation responses, respectively); however, the physiological significance of this transphosphorylation is questionable [7]. Purified phospho-NtrB protein also has been reported to phosphorylate the CheY protein [7]. In the current study, we tested the hypothesis that chemotaxis to PTS substrates is mediated by transphosphorylation between a PTS component and the chemotaxis system. Our preliminary findings have been reported previously [8,9].

## 2. Materials and methods

### 2.1. Bacterial strains

*S. typhimurium* strains LJ2028 (*ptsH fruR*::Tn*10*) and LJ2046 (*ptsH*) were provided by M. Saier. *E. coli* strains DS165 (*ptsI crr*) [10] and MM335 (wild-type for chemotaxis and PTS) were obtained from S. Roseman and M. Manson, respectively.

### 2.2. Protein purification

The CheW, CheA, CheY, HPr, Enzyme I and Enzyme II$^{mtl}$ proteins were purified using minor modifications of published techniques [11–16]. Hydrophobic interaction high performance liquid chromatography (Synchropak) replaced or was added to the final step in the purification of CheW, CheA, CheY, and Enzyme I. Phosphoenolpyruvate carboxykinase was partially purified (free of PEP carboxylase, NADH oxidase and pyruvate kinase) by gel filtration (Sephadex G-100) of a 40–60% ammonium sulfate cut from the crude extract. [$^{32}$P]PEP was synthesized with phosphoenolpyruvate carboxykinase and purified as described by Mattoo and Waygood [17]. Overexpression vectors pDV4 (*cheA cheW*; [18]), pRL22 (*cheY*; [13]), pDS20 (*ptsH, ptsI*, structural genes for HPr and Enzyme I; [10]) and pCAS2.0 (Enzyme II$^{mtl}$ clone; [19]) were used to produce adequate quantities of the proteins of interest for this study.

### 2.3. Phosphorylation experiments

Reaction mixtures for phosphate exchange between PTS proteins and chemotaxis proteins contained 50 mM Tris-HCl (pH 8.9), 10 mM

*Corresponding author. Fax: (1) (909) 824 4035.
E-mail: blTaylor@ccmail.llu.edu

**Present address:* University of Minnesota Medical School, Department of Laboratory Medicine and Pathology, Box 609, UMHC, 420 Delaware St., SE, Minneapolis, MN 55455, USA.


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MgCl, 50 mM KCl, 8 mM Dithiothreitol, 60 μM EDTA, 200 μM [$^{32}$P]PEP (450 mCi/mmol) or 1 mM [γ-$^{32}$P]ATP (50 mCi/mmol) and proteins at concentrations described in the figure legends. After incubation at 25°C for 10 min, the reactions were stopped by the addition of SDS sample buffer. Samples were run on 8% Tris-glycine SDS polyacrylamide gels [20] to separate CheA from Enzyme I, or 10% Tris-Tricine polyacrylamide gels [21] to separate CheY from HPr. After autoradiography, bands were excised and counted to quantitate phosphorylation.

Cycling and phosphorylated PTS components were prepared by preincubating all proteins for 5 min in the presence of 200 μM PEP followed by the concomitant addition of [γ-$^{32}$P]ATP and 200 μM mannitol (cycling) or [γ-$^{32}$P]ATP alone (phosphorylated state). PEP was omitted from preincubations to prepare nonphosphorylated PTS components. When applicable, large HPr:CheA ratios were achieved by increasing HPr to 65 μM and by decreasing CheA to 0.42 μM. The source of the cell extract was *E. coli* DS165 (pDS20) cells overexpressing the Hpr and Enzyme I proteins [10]. The cell extract consisted of the supernatant obtained from the cells disrupted in a French press and centrifuged at 8,000 × *g* to remove cell debris.

## 3. Results

The CheA, CheW, CheY proteins and the PTS proteins for mannitol transport were incubated with [γ-$^{32}$P]ATP or [$^{32}$P]PEP in the presence and absence of cell extract to determine whether transphosphorylation occurred between the proteins. Enzyme I and HPr in *E. coli* DS165 (pDS20) were expressed at levels approximately 4-fold above that of wild-type *E. coli* MM335 (as determined by activity assays). These proteins were easily identified in whole cell extracts as [$^{32}$P]PEP phosphorylated products that comigrated with purified Enzyme I and HPr proteins, respectively. Purified CheA protein was not phosphorylated by [$^{32}$P]PEP (Fig. 1, lane 1). To determine whether CheA is phosphorylated by PEP in the presence of additional cellular components, purified CheA (8 μM) was incubated with whole cell extract (Fig. 1, lane 3) under conditions which readily phosphorylate Enzyme I and HPr (Fig. 1, lane 2). No phospho-CheA product was observed. The threshold for identifying a phosphorylated band in the autoradiograms was less than 100 dpm; this corresponds to a minimum of 0.1 pmol of phospho-CheA. This result translates to a threshold of one phospho-CheA for every 1,290 unphosphorylated CheA proteins under assay conditions. Transphosphorylation at levels below this threshold would be of doubtful significance for signal transduction. The addition of cell extract to purified CheA

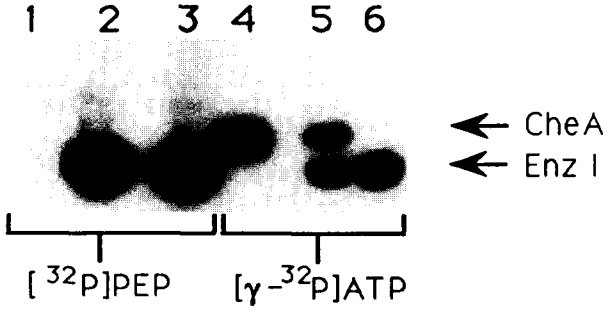


Fig. 1. Phosphate exchange between PTS proteins and chemotaxis proteins. Reaction mixtures and conditions are described in section 2; 8 μM CheA and 2 mg/ml cell extract were included where applicable. Gel lanes: 1, [$^{32}$P]PEP and CheA; 2, [$^{32}$P]PEP and cell extract; 3, [$^{32}$P]PEP, CheA and cell extract; 4, [γ-$^{32}$P]ATP and CheA; 5, [γ-$^{32}$P]ATP, CheA and cell extract; and 6, [γ-$^{32}$P]ATP and cell extract.

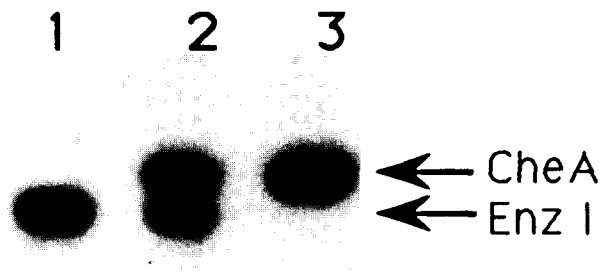


Fig. 2. Phosphorylation of Enzyme I by ATP in the presence of dialyzed cell extract. Lanes: 1, [γ-$^{32}$P]ATP and cell extract; 2, [γ-$^{32}$P]ATP, CheA and cell extract; and 3, [γ-$^{32}$P]ATP, CheA, cell extract and 1 mM PEP. Incubation conditions were the same as described in Fig. 1.

lowered the quantity of phosphorylated CheA produced from [γ-$^{32}$P]ATP (Fig. 1: compare lanes 4 and 5). This was expected because the cytosol contains phosphatases, ATPase and other kinases that decrease CheA phosphorylation. Unexpectedly, [γ-$^{32}$P]ATP phosphorylated Enzyme I in the presence of cell extract (Fig. 1, lane 5). To determine whether this phosphorylation required CheA, purified CheA was omitted from the incubation, decreasing the CheA concentration from 8 μM to less than 80 nM (from cell extract). The level of Enzyme I phosphorylation increased (Fig. 1, lane 6). Thus, CheA is not necessary for ATP-dependent phosphorylation of Enzyme I.

To test whether phosphoryl transfer occurred via a small molecule such as PEP or acetyl phosphate, the cell extract was dialyzed (3000 MW cutoff) against 50 mM Tris-1 mM dithiothreitol buffer (pH 8.0) and the incubations were repeated. [γ-$^{32}$P]ATP still labeled Enzyme I in the dialyzed cell extract (Fig. 2, lane 1). The addition of non-radioactive PEP to a concentration of 1 mM inhibited Enzyme I labeling (Fig 2, lane 3) but interestingly increased CheA labeling (compare with lane 2). Fox et al. reported that acetate kinase can phosphorylate Enzyme I in the presence of PEP, GTP or ATP [22]. To test whether this might also be responsible for the ATP-dependent Enzyme I phosphorylation, an assay mixture similar to that in Fig. 2, lane 3 was titrated with cold GTP. GTP inhibited the ATP-dependent phosphorylation of Enzyme I (approximately 95% at 10:1, GTP/ATP), but increased the level of CheA phosphorylation by [$^{32}$P]ATP (data not shown). This is consistent with ATP-dependent transphosphorylation through acetate kinase to Enzyme I in whole cell extracts.

We observed no evidence of direct or indirect phosphorylation of the CheA protein, or of another chemotaxis protein, by PEP, HPr or Enzyme I. We also investigated whether the unphosphorylated HPr protein inhibited phosphorylation of the CheA or CheY protein. The rates of [γ-$^{32}$P]ATP-dependent phosphorylation of CheA and CheY in the presence of the PTS components were studied. Assay mixtures included CheW, CheA, Enzyme I, HPr, Enzyme II$^{mtl}$, and CheY (when applicable). Over a 2-min interval, there was no significant difference in the rate of CheA (data not shown) or CheY phosphorylation (Fig. 3) when PTS components were unphosphorylated, cycling, or phosphorylated. The experiment was repeated and crude cell extract was added to the assay mixture to supply any intermediate that might be required for PTS regulation of CheA phosphorylation. The phosphorylated CheY protein was unstable in the presence of cytosol, reaching a maximum level of

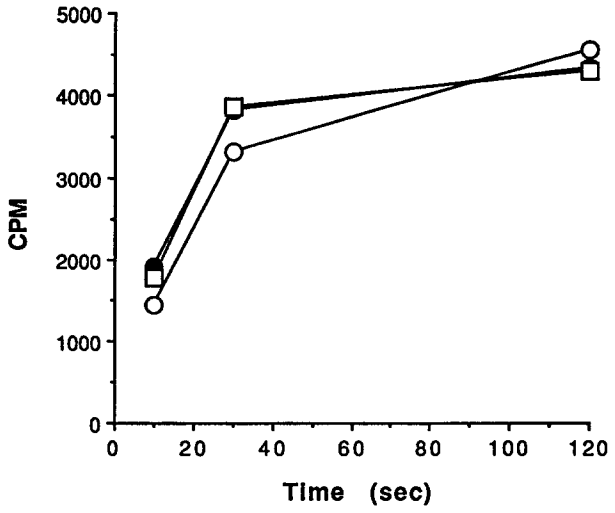


Fig. 3. Effect of PTS on the rate of phosphorylation of CheY by ATP in a reconstituted system. Reactions contained purified CheY (12 $\mu$m), CheA (8.6 $\mu$M), CheW (9.2 $\mu$M) and the PTS proteins, Enzyme I (0.5 $\mu$M), HPr (3.3 $\mu$M), Enzyme $II^{mtl}$ (0.028 $\mu$M) in cycling (○), nonphosphorylated (●) or phosphorylated (□) states as described in the text. The ATP labeling conditions are described in section 2.

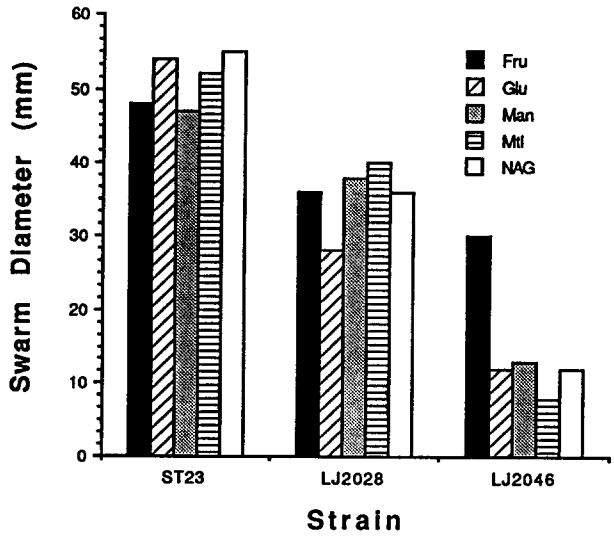


Fig. 5 Restoration of PTS chemotaxis by FPr in *S. typhimurium* HPr mutants. Bacterial culture (5 $\mu$l) was injected into the center of minimal media semisoft swarm plates supplemented with 100 $\mu$M of the designated PTS sugar. The plates were incubated at 30° C for 24 hours and the diameters of the swarms or areas of growth were measured. Strains (from the left): ST23 (*che*$^+$ *pts*$^+$ *hisF thyA*), LJ2028 (*ptsH fruR*::Tn*10*), and LJ2046 (*ptsH*). Abbreviations: Fru, fructose; Glu, glucose; Man, mannose; Mtl, mannitol; NAG, *N*-acetylglucosamine.

phosphorylation by 30 s. The ATP-dependent phosphorylation of Enzyme I was evident within 2 min, particularly when PTS components were in the nonphosphorylated state (Fig. 4).

Since the intracellular ratio of HPr:CheA is less than 60 [23] and the ratio of HPr:CheA in these experiments was as high as 152, it is likely that we would have detected any inhibition of CheA by HPr that was of physiological importance. We confirmed that the HPr and the CheA proteins do not physically interact using fluorescence polarization. HPr which was tagged with the fluorescent probe 1,5-IAEDANS as described by Hildenbrand et al. [24] retained 64% of its native activity. Rotational correlation times of the tagged HPr (estimated from Perrin plots) were unchanged by the presence of the CheA protein (ratio HPr/CheA = 1:1).

In view of the finding that the HPr protein does not phosphorylate the CheA protein or inhibit autophosphorylation of CheA, we reexamined evidence supporting HPr as the PTS component that interacts with the chemotaxis pathway. Specifically, we repeated in *S. typhimurium* the experiments previously reporting that the HPr protein is required for chemotaxis

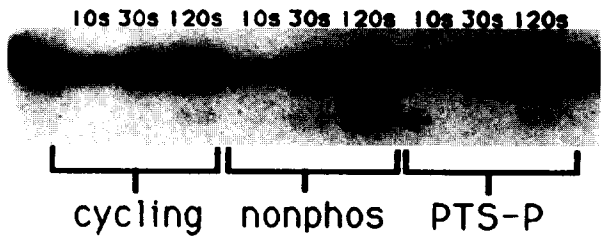


Fig. 4. Effect of PTS and cell extract on the phosphorylation of CheA by ATP. Assay conditions are the same as described in Fig. 3, with the addition of cell extract (2 mg/ml). Enzyme I, HPr, and Enzyme $II^{mtl}$ proteins were in the phosphorylated (PTS-P), cycling or nonphosphorylated (nonphos) states as designated. Enzyme I phosphorylation (see arrow) is evident within 2 min when the preincubated PTS is in the nonphosphorylated state.

but not for transport in *E. coli* [5,6]. PTS chemotaxis on swarm plates corresponded with expression of the FPr protein in *S. typhimurium ptsH* cells deficient in the HPr protein, (Fig. 5). The diameters of swarms relative to those of wild-type cells (strain ST23) were approximately 70% for the PTS sugars tested in cells of *S. typhimurium* LJ2028 (*ptsH fruR*::Tn*10*) which express FPr constitutively. The relative diameter of swarm size was approximately 20% for cells of strain LJ2046 (*ptsH*) for the sugars tested, except for fructose which was 60%. The FPr protein is expressed in LJ2046 cells only when the inducer fructose is present. Thus, restoration of PTS chemotaxis and transport in *S. typhimurium* does not require overexpression of the FPr protein as it does in *E. coli*.

## 4. Discussion

The data demonstrate that ATP can phosphorylate Enzyme I in the presence of cell extract, but suggest that this is not through CheA. The inhibition of this transphosphorylation by PEP or by GTP, and the persistence of activity after dialysis is consistent with the intermediary being acetate kinase. If so, this is the first evidence that phosphoryl transfer from ATP to Enzyme I may be significant at constitutive levels of cytosolic acetate kinase. Acetyl phosphate, a substrate of acetate kinase, can phosphorylate CheY [25]. Thus, it had been suggested that PTS-derived fluctuations in acetyl phosphate might affect CheY-phosphate levels and thereby control PTS chemotaxis. However, mutants incapable of synthesizing acetylphosphate remain capable of normal PTS transport and chemotaxis in spatial and temporal assays [26,27].

Our results indicate that the PTS components do not directly or indirectly phosphorylate the CheA or CheY proteins. Under conditions where Enzyme I and HPr were phosphorylated,

phosphoryl transfer was below the threshold of resolution for CheA (phospho-CheA/CheA = 1:1,290) and for CheY (phospho-CheY/CheY = 1:1,800). Thus, it seems likely that a component of the PTS interacts with the CheA (or CheY) protein to inhibit or activate the rate of phosphorylation. The data presented argue against a role for HPr in the regulation of CheA phosphorylation. The alternative hypothesis is that Enzyme I interacts directly or indirectly with the CheA (or CheY) protein. While this manuscript was in preparation, Lengeler and his associates [28] reported that the ATP- dependent phosphorylation of CheA is inhibited by the phosphorylated Enzyme I but not by unphosphorylated Enzyme I. This inhibition becomes significant at Enzyme I/CheA ratios higher than 1:1, and would not have been observed at the relative concentrations used in our study.

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