

Genetic Engineering of the Phosphocarrier Protein NPr of the *Escherichia coli* Phosphotransferase System Selectively Improves Sugar Uptake Activity^{*[S]}

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Background: HPr and NPr phosphorylate different proteins of the PTS system with negligible cross-reactivity.

Results: Transplanting a few residues from HPr into NPr transferred HPr-like specificity with wild type efficiencies and differential activities.

Conclusion: Few changes were needed to produce a functional chimera, showing a straightforward instance of protein evolution.

Significance: These results further our understanding of the protein-protein recognition mechanisms regulating protein phosphorylation.

The *Escherichia coli* phosphoenolpyruvate:sugar phosphotransferase system (PTS) in prokaryotes mediates the uptake and phosphorylation of its numerous substrates through a phosphoryl transfer chain where a phosphoryl transfer protein, HPr, transfers its phosphoryl group to any of several sugar-specific Enzyme IIA proteins in preparation for sugar transport. A phosphoryl transfer protein of the PTS, NPr, homologous to HPr, functions to regulate nitrogen metabolism and shows virtually no enzymatic cross-reactivity with HPr. Here we describe the genetic engineering of a “chimeric” HPr/NPr protein, termed CPr14 because 14 amino acid residues of the interface were replaced. CPr14 shows decreased activity with most PTS permeases relative to HPr, but increases activity with the broad specificity mannose permease. The results lead to the proposal that HPr is not optimal for most PTS permeases but instead represents a compromise with suboptimal activity for most PTS permeases. The evolutionary implications are discussed.

Often, paralogous proteins in a cell play different roles, particularly when sequence divergence is substantial. For most proteins, it is not known which evolutionary pathways gave rise to this functional distinction. By studying their evolution, structure, and function through genetic engineering and bioinformatic analyses, we hope to understand the complex relationships between representative paralogs. Protein engineering

approaches, such as the ones performed in this study, illustrate the utility of such studies.

We have compared two structurally similar homologous proteins, HPr and NPr, of the *Escherichia coli* phosphoenolpyruvate-dependent phosphotransferase system (PTS)³ (1). These proteins are paralogs but have different functions; HPr is an essential phosphoryl transfer protein in the sugar-transporting PTS (2), whereas NPr is part of a distinct nitrogen (Ntr) regulatory phosphoryl transfer chain that influences nitrogen metabolism by regulating gene expression (3). NPr cannot substitute for HPr in promoting sugar transport and phosphorylation, and HPr cannot substitute for NPr in coordinating nitrogen metabolism with carbon metabolism (4). These two phosphoryl transfer chains are as shown in Scheme 1.

E. coli possess a complex PTS with numerous constituent proteins, many of which are still functionally uncharacterized. For example, in *E. coli*, there are five Enzyme I (EI) proteins, six HPr homologs, and 21–22 Enzyme II (EII) complexes (5). The EII complexes generally consist of EIIA, EIIB, and IIC, but the EII complexes of the mannose family additionally have an IID constituent. All are required for function.

As described in the Transporter Classification Database (TCDB) (6, 7), there are three evolutionarily distinct families of sugar-transporting PTS EII complexes: the glucose-fructose-lactose (GFL) superfamily, the ascorbate-galactitol (AG) superfamily, and the mannose-fructose-sorbose (Man) family (8). Additionally, there is one nontransporting EII complex: the dihydroxyacetone (DHA) family (9, 10). The GFL superfamily contains the transport pathways for glucose, α -glucosides, β -glucosides, fructose, mannitol, lactose, *N,N'*-diacetylchito-

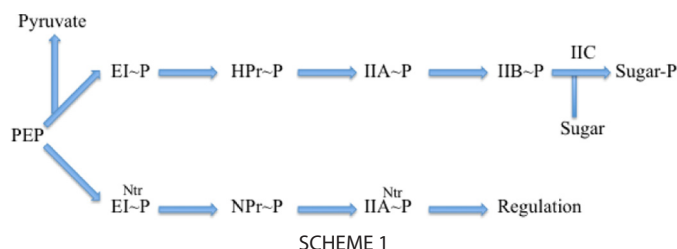
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[S] This article contains supplemental Tables S1–S3 and Figs. S1–S3.

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³ The abbreviations used are: PTS, phosphotransferase system; EI, Enzyme I; EII, Enzyme II; GFL, glucose-fructose-lactose; AG, ascorbate-galactitol; Man, mannose-fructose-sorbose; DHA, dihydroxyacetone; IPTG, isopropyl-1-thio- β -D-galactopyranoside.



biose, and glucitol (11). The AG superfamily includes the proteins specific for galactitol and L-ascorbate (8, 12). The mannose family transporters transport a wide variety of aldo- and ketohexoses (13). The sole substrate of DHA family Enzyme II is DHA (10).

Transporters within these superfamilies are generally quite specific for their target sugars, but as noted above, the Man family consists of members with broad specificity. The *E. coli* EII^{Man} transports all hexoses of the D-glucose configuration with promiscuity at the 2 position (14). They are the only PTS proteins in *E. coli* known to appreciably transport mannose, glucosamine, and the nonmetabolizable sugar analog, 2-deoxy-D-glucose, and they also transport glucose as their preferred substrate as well as *N*-acetyl glucosamine and *N*-acetyl mannosamine as low-affinity substrates (13).

One well characterized PTS phosphoryl transfer chain used by *E. coli* and many other bacteria uses phosphoenolpyruvate and IIA^{Ntr}. It functions in nitrogen regulation and is not known to provide phosphoryl groups to any of the sugar-transporting EII complexes. The functions of the nitrogen regulatory branch of the PTS are not as well understood as the sugar-translocating branch. However, regulatory functions include control of nitrogen metabolism (4, 15–17), lipid A biosynthesis (18), K⁺ transport (19), β -glucoside utilization (20), toluene degradation (21), pathogenesis (22), and biofilm formation (23). IIA^{Ntr}, and perhaps NPr, are transcription factors for many operons, particularly those involved in nitrogen metabolism. These two PTS phosphoryl transfer chains show less than 1% overall enzymatic cross-reactivity (4).

In the analyses reported here, genetic engineering was used to create a “chimera” of HPr and NPr, derived from the *E. coli* *npr* gene. This chimera includes the NPr backbone but with 14 site-specific amino acid substitutions, rendering it more similar to HPr. Analysis of this chimeric NPr protein, which we will refer to as CPr14, provided clues as to how the distinct specificities of HPr and NPr arose. It also revealed the surprising fact that HPr is not optimal for all sugar-transporting Enzyme II complexes.

CPr14 proved to be more active than the native HPr when assayed for IIA^{Man} activity *in vivo*. Growth, fermentation, and transport assays led to the conclusion that the synthetic chimera, CPr14, is superior to HPr with this one Enzyme II complex. CPr14 functioned less effectively than HPr with other EIIs, indicating that wild type HPr is not optimized for its activities with IIA^{Man}. It may instead have suboptimal activity with some of its IIA interaction partners, thus representing an attempt at average optimization for interaction with several of the different IIA partners.

EXPERIMENTAL PROCEDURES

Plasmids and Strains—The pZE12 plasmid was used in the majority of the experiments, although pBAD24 was also used. pZE12 contains a *lac* promoter that is induced by IPTG (24). pBAD24 contains a promoter that is induced by L-arabinose (25, 26). The strains employed were derivatives of BW25113 (27). See supplemental Table S1.

Construction of NPr Variant Proteins—To determine whether converting interfacial residues of NPr into those of HPr would confer HPr function on NPr, we chemically synthesized and constructed the entire gene (supplemental Fig. S2). This was achieved using six oligonucleotides, three of which code for the desired amino acid changes. The synthetic gene (CPr14) was cloned into vector pZE12 (24). During this process, we also preserved, as separate clones, two constructs with either the first 6 (CPr6) or the last 8 (CPr8) amino acid replacements.

HPr and FPr Double Knock-out—Growth analyses on the HPr/FPr knock-out (2 Δ) confirmed the results of the study of Feldheim *et al.* (28), which showed that these cells are unable to utilize any PTS sugar. It should be noted that the knock-out of FPr involved knocking out *fruB*, which encodes the FPr-IIA^{Fru} fusion protein. Studies on M9 minimal medium sugar plates showed that the 2 Δ phenotype is rescued by expression of HPr or CPr14 but not by expression of NPr (supplemental Table S2). The complementation analyses were confirmed by MacConkey fermentation analyses on glucose, mannose, and mannitol (supplemental Table S3).

Cloning HPr and NPr—The *ptsH* (*i.e.* HPr) structural gene was amplified from the BW25113 genomic DNA using primers *ptsH*-Nco and *ptsH*-Hind (supplemental Table S1). The PCR products were digested with NcoI and HindIII, gel-purified, and then ligated into the same sites of pBAD24 (26), yielding pBAD24-*hpr* where *hpr* expression is under the control of the *araBAD* promoter. To induce expression of *hpr* in pBAD24-*hpr*, 1 mM L-arabinose was added to the medium. Similarly, the *ptsN* (*i.e.* *npr*) structural gene (encoding NPr) was amplified from the genomic DNA using primers *npr*-Kpn and *npr*-Hind (supplemental Table S1) and then cloned between KpnI and HindIII of pZE12 (24).

Aerobic Growth Experiments in Liquid Media—All growth experiments were performed in liquid M9 minimal media (1 mM IPTG and 100 μ g/ml ampicillin); all incubations were in a 37 °C water bath. Sugars were added at 0.6% unless otherwise specified. Cultures from strains 2 Δ HPr, 2 Δ NPr, and 2 Δ CPr14 were first grown from single colonies in liquid LB with ampicillin overnight. LB cultures (0.5 ml) were used to inoculate 24 ml of M9 minimal medium, and cultures were grown overnight. One milliliter of overnight M9 minimal medium was added to 24 ml of fresh M9 medium for the HPr and CPr14 strains at an optical density reading (600 nm) close to 0.2. For the NPr strain, 14 ml of overnight M9 minimal medium culture was added to 11 ml of fresh M9 minimal medium for an A_{600} reading close to 0.2.

HPLC Analysis—A carbohydrate column capable of discrimination between the six carbon sources (glucose, mannose, mannitol, galactitol, sorbitol, and xylose) was used on an HPLC

machine. The machine was calibrated using concentrations of 1.5, 1.0, 0.5, or 0.1 g/liter for each carbon source. Carbohydrates in the supernatants were analyzed by HPLC using the following components: Waters equipment; 600E quaternary bomb; 717 automatic injector; and 2410 refraction index detector. An Aminex HPX 87P column (Bio-Rad) was connected. Running conditions for the mobile phase were as follows: H₂O; flow, 0.6 ml/min; and temperature, 85 °C.

Radioactive Uptake Assays—CPr14, HPr, and NPr 2Δ cells were grown overnight in 5 ml of LB with 0.2% mannitol, 1 mM IPTG, and 100 mg/ml ampicillin in a 37 °C shaking water bath. One milliliter of the overnight culture was added to 35 ml of LB with 0.2% mannitol, 1 mM IPTG, and 100 mg/ml ampicillin. This was incubated in a 37 °C shaking water bath until the cells reached log phase (A_{600} of ~0.5). Cells were washed three times by centrifugation at 10,000 rpm at 0 °C for 5 min and resuspended in 20 ml of cold M9 salts solution. The final resuspension was performed using 10 ml of cold M9 salts solution. Cells were then diluted to an A_{600} of 1 ± 0.1 with an M9 salts solution. An aliquot of 4.5 ml of cells was then given 0.5 ml of LB, and the suspension was incubated at room temperature for 10 min. Cells were given 50 μl of a 1 mM radioactive sugar solution at 5 microcuries/μM for a final concentration of 10 μM sugar at 5 microcuries/μM. Cells were incubated at room temperature or at 37 °C with shaking, and samples of 100 μl each were removed at the designated time intervals and passed through 0.45-μm Whatman filters. The filters were washed three times with 10 ml of a cold M9 salts solution. Filters were then dried under a heat lamp for 10 min and dissolved in 10 ml of scintillation fluid NA.

Modeling of Proteins—Using four known structures (HPr, HPr-EI, HPr-EIIA^{glc}, HPr-EIIA^{mtl}, and HPr-EIIA^{man}; Protein Data Bank (PDB): 3EZA, 1GGR, 1J6T, and 1VRC, respectively) as templates, structural modeling of CPr14 and NPr was conducted using the methods align2d and automodel from MODELLER9v7 (29). NPr has 26.4% identity to HPr, and the partial structure suggests an equivalent folding. Furthermore, the 14th mutation in CPr takes this identity to 41.8%. Those models were assayed for quality using the DOPE (30) scores method of MODELLER9 version 7 (supplemental Fig. S3). Before the docking procedure, the HPr structure and NPr CPr14 models were structurally aligned to HPr in the corresponding crystal using Swiss PDB-viewer v4.0.1 (31). All combinations of HPr, NPr, CPr14 with EIIA^{glc}, HPr-EIIA^{mtl}, and HPr-EIIA^{man} were constructed for a total of nine hybrid crystal models.

Hex6.1+CUDA (32) were used for molecular docking with default parameters except for the following: correlation type, shape + electrostatics; compute device, central processing units + graphics processing units; post processing, molecular mechanics minimization; twist range, 180, and distance range, 10 Å. This is to limit the search space to conformations close to the crystal model. As a control for the docking procedure, the root mean square deviation of the HPr crystal against the docked model was calculated. It was less than 2 Å for all models, showing that hex6.1 is capable of reproducing known interactions and conformations.

RESULTS

Identification of Specificity Residues within the Contact Surfaces—Based on the experimental structure of the HPr-EIIA^{mtl} complex (Cornilescu *et al.* (40); PDB ID: 1J6T), 14 HPr residues at the interaction surface were identified as candidates for specificity determination (Fig. 1). These residues are all well conserved among the HPrs and among the NPrs but not between these two functionally distinct families of homologs. These NPr residues were converted into those found in the HPrs based on a multiple alignment of HPrs with NPrs. Three mutant chimeric *npr* genes were synthesized using overlap extension PCR. One had all 14 mutations (CPr14); one had the first six N-terminal mutations (CPr6); and one had the last 8 C-terminal mutations (CPr8) (Fig. 1).

Construction of the Structural Models for NPr and CPrs—Until now, a model for the NPr protein did not exist despite the partial structure of NPr determined by Li *et al.* (33) and considering a 0.33 identity between both proteins for *E. coli*. We decided to use a homology modeling strategy for the construction of the NPr model. The MODBASE server (34) was used for modeling an NPr structure using an HPr template (PDB 1POH).

The computed models were tested with the different types of indexes provided by the MODBASE server (E-value, the final model score, and ModPipe Protein Quality Score). All models selected and used in the next analysis passed the reliability tests provided by the MODBASE server (data not shown).

The resulting models highlight the differences between the interacting surfaces of HPr and NPr. Of note are the different protrusions, crevices, and charge distributions and the formation of a cavity very similar to that observed in the HPr model (Fig. 1).

Experimental HPr PTS Complexes and Modeled Complexes for CPr14—Protein modeling analyses were performed to compare the experimental complexes of HPr and its IIA proteins with those predicted for CPr14 and the IIA proteins (Fig. 2). It is noteworthy that the complexes for HPr are similar to those predicted for CPr14. Both show similar calculated ΔG binding values and predicted distances between the two phosphorylatable histidines on the IIAs and HPr or CPr14.

In Vivo Functional and Complementation Analyses—Experiments in this study were performed in the *E. coli* BW25113 parental strain with the *fruB* (FPr-IIA) and *ptsH* (HPr) genes deleted, hereafter referred to as 2Δ cells (35). Strains were created by electroporation of the HPr, NPr, CPr14, CPr6, or CPr8 genes in the pZE12 vector (inducible by IPTG) into the 2Δ cells. A detailed description can be found in the supplemental data.

Liquid growth studies were performed using 2Δ-HPr, 2Δ-NPr, and 2Δ-CPr14 cells in glucose, mannitol, *N*-acetylglucosamine, mannose, glucosamine, and fructose M9 minimal media (Fig. 3 and Table 1). 2Δ-HPr and 2Δ-CPr14 cells grew in all these media, whereas the 2Δ-NPr cells did not show significant growth in any medium. Interestingly, for the sugars transported solely by II^{Man}, mannose, glucosamine and fructose, 2Δ-CPr14 cells consistently outgrew the 2Δ-HPr cells, suggesting that CPr14 is more efficient for sugar uptake via the mannose Enzyme II complex. Growth rates and doubling times in

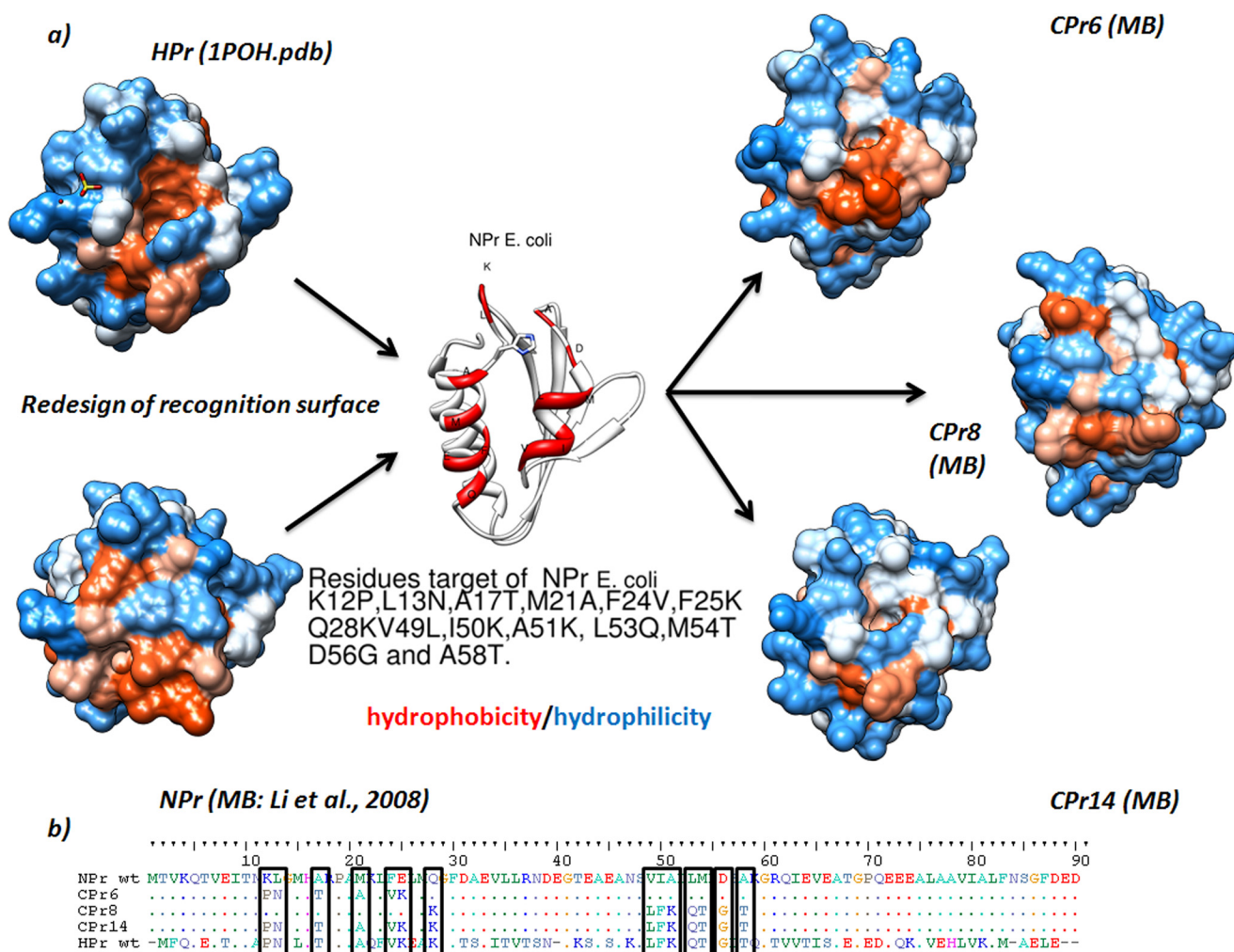


FIGURE 1. **Redesign of the recognition surface of NPr protein.** *a*, different recognition surfaces of HPr (WT), NPr (WT), and the various CPrs in the same spatial orientation. Coordinates for NPr and the three chimeric proteins were obtained by comparative modeling from the MODBASE (MB) database (Pieper *et al.* (34)). *b*, alignment of NPr, HPr and CPrs; mutated residues are highlighted by rectangles.

each medium are listed in Table 1. It should be noted that fructose is utilized much more efficiently by CPr14 cells than by HPr cells. Because the fructose-specific Enzyme II complex is nonfunctional, fructose enters the cell only via the mannose system. Because fructose is a poor substrate relative to glucose, mannose, and glucosamine, we can explain this observation by assuming that some intracellular fructose-6-P is lost via phosphatase activities. Anaerobic growth in M9-ascorbate was also examined, with CPr14 performing slightly worse than HPr. Insignificant growth of 2Δ cells expressing NPr was observed (supplemental Fig. S1).

The two other CPr variants with partial substitutions were tested for growth in parallel with the HPr-, NPr-, and CPr14-producing strains in M9 medium with seven different PTS sugars and one non-PTS sugar (xylose). When compared with CPr14, CPr6 restored a significantly lower level of growth, with a more robust rescue in glucose, *N*-acetylglucosamine, and mannose. The CPr8 variant was unable to restore growth in the genetic background of the 2Δ cells (Fig. 4).

HPLC was used to determine the preferential use of the different carbon sources in a mixture of five different PTS sugars in M9 medium (Fig. 5). The order of consumption for the

BW25113 parental 2Δ-HPr and 2Δ-CPr14 cells was approximately the same: glucose > mannose > xylose = mannitol > galactitol > glucitol. Sugar usage by 2Δ-CPr6 cells was in agreement with the data obtained for growth in liquid media (Fig. 4). Consistent with earlier findings (36), other sugars, PTS-dependent or otherwise, were only utilized after glucose had been depleted from the medium. Interestingly, xylose (a non-PTS sugar) was used before the PTS polyols, mannitol, galactitol, and glucitol. 2Δ-NPr cells were unable to grow in or utilize any of the sugars included in the HPLC analysis (data not shown). It is worth mentioning that to the best of our knowledge, this is the first report determining the sequence of PTS sugar consumption in the presence of glucose for *E. coli*.

Radioactive uptake assays performed with 2Δ-HPr, 2Δ-NPr, and 2Δ-CPr14 cells using [¹⁴C]glucose and 2-deoxy-D-[¹⁴C]-glucose showed that the 2Δ-HPr cells took up glucose more readily than the 2Δ-CPr14 cells, but as shown in Fig. 6, the 2Δ-CPr14 cells took up 2-deoxy-D-glucose better. Because 2-deoxy-D-glucose, transported only by the mannose Enzyme II complex, is nonmetabolizable, the accumulation observed must be a reflection of the uptake rate unaffected by subsequent metabolism. 2Δ-CPr14 cells plateaued at ~2000–2500 cpm,

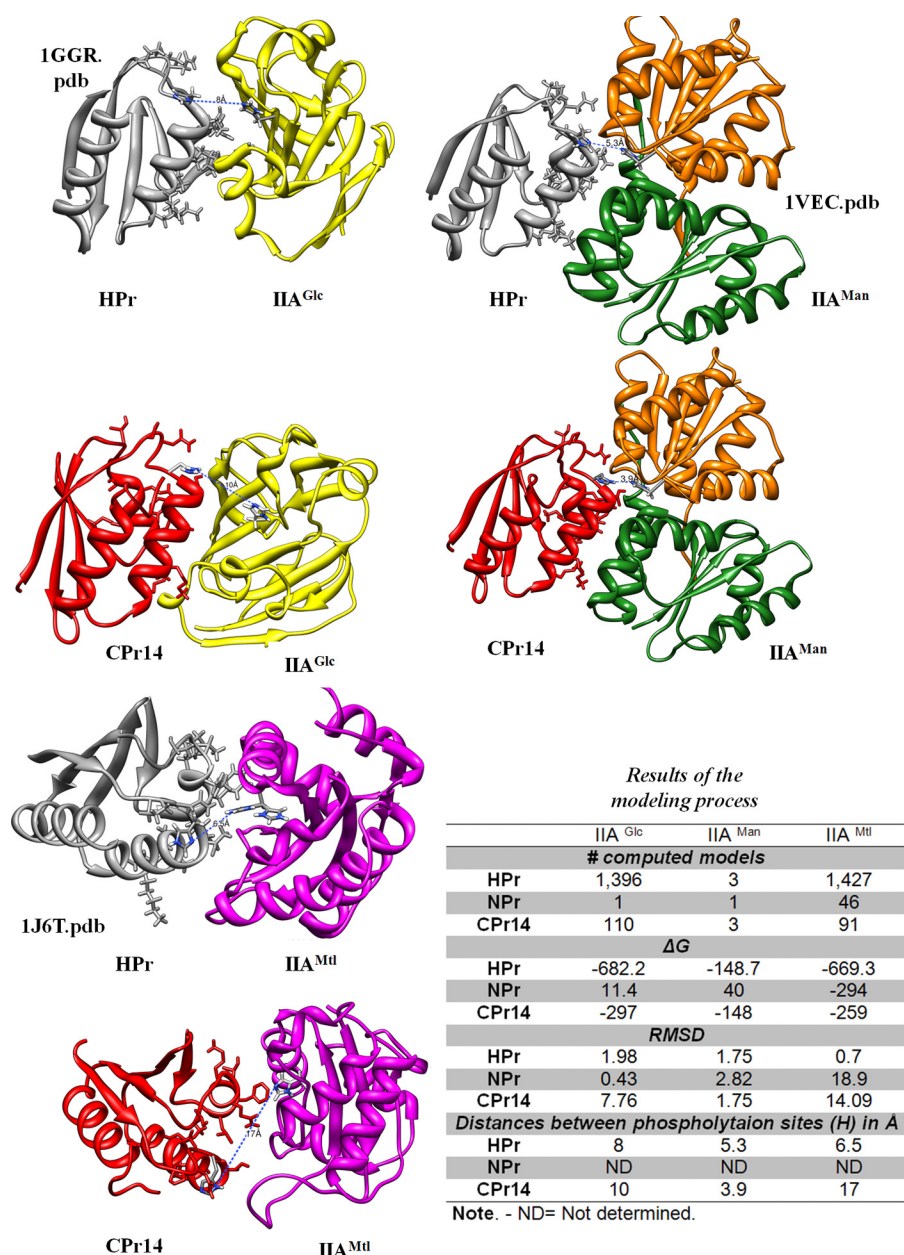


FIGURE 2. Comparison among experimental complexes (HPr and its partner IIA proteins) and the modeling complexes (CPr14 and the IIA proteins). Only side chains of residues on the recognition surfaces are shown. For each of the nine dockings, the table shows the number of calculated models that had less energy than the default threshold (or the best model if none were better than the threshold), the ΔG of binding for the best model (in Hex units), the root mean square deviation (RMSD) of the best docking model against the crystal model, and the distance between phosphorylation sites.

whereas 2Δ-HPr cells plateaued at ~1500 cpm. These results confirm that II^{Man} prefers CPr14 to HPr.

DISCUSSION

Uses and Limits of an Interaction Model Based on Homologous Complexes—The remarkable efficiency with which CPr14 was able to allow for growth in the absence of HPr indicates that this chimera retained sufficient structure and stability to allow function. Although none of the 14 residues modified seemed to be critical to the structure of the protein, it is possible that they reduced the stability and normal function of NPr. The success of these experiments can be attributed to the fact that HPr and NPr are structurally similar homologs. Indeed, similar results

were obtained when sequence changes introduced diversity present in other sets of homologous proteins (37).

Comparison between the Models and Experimental Structures—In the phosphorylation chain of the PTS, HPr involves the formation of two interfaces (EI and IIA^{Glc}). It is noteworthy that one of the key interactions with the interface between HPr and its partners was presented in the models of our mutants (Fig. 1). Despite the fact that both HPr and NPr have very similar scaffolds (33), HPr has a convex region (helices 1 and 2) that serves as a complementary fit in the protein-protein interaction with EI and IIA^{Glc} (38, 39). This common convex binding surface (for EI and IIA^{Glc}) has a central hydrophobic core surrounded by a ring of polar and positively

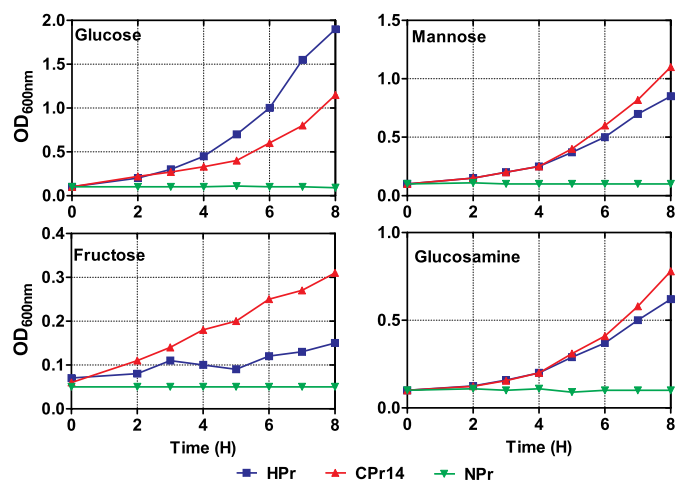


FIGURE 3. Comparative growth of 2Δ-BW25113 ($\Delta ptsH$, $\Delta fruB$; 2Δ) with HPr, NPr, or CPr14 expressed from pZE12. Liquid M9 minimal medium was used with one of four different carbon sources: glucose, mannose, glucosamine, or fructose, each at 0.2% with 1 mM IPTG at 37 °C. Experiments were performed in triplicate, and the results presented are the average values. Standard deviations were too small to be displayed. OD, optical density.

TABLE 1

Rates of growth of 2Δ-HPr and 2Δ-CPr14 cells in liquid M9 sugar media

Doubling times were calculated using the data from the logarithmic growth phases of the cells, as indicated by a linear growth phase in a logarithmic graph (A_{600}) as a function of time (Fig. 3). Growth rate is expressed as the percentage of the wild type rate in glucose medium.

M9 minimal medium	Growth rate		Doubling time	
	HPr	CPr14	HPr	CPr14
	%		h	
Substrate				
Glucose	64	45	1.4	1.9
Mannitol	38	37	2.2	2.2
Sorbitol	40	37	2.1	2.2
Mannose	32	42	2.5	2.0
Glucosamine	31	41	2.5	2.0
Fructose	13	19	5.5	4.0

charged residues. In our protein models, we can see that this attribute is shared by HPr and mutants CPr14 and CPr6 but is absent in CPr8 and NPr. It is possible that this convex surface is needed to help the formation of the complex with EI and IIA^{Glc}. Although we have experimental evidence supporting the notion that CPr6 and CPr14 have the capacity to restore growth in the complementation assays, it is necessary to compare the models with experimental data obtained with structural studies (NMR or x-ray crystallography).

In Silico Analyses and Experimental HPr PTS Complexes versus Modeled CPr14 Complexes—Protein modeling and docking analyses were performed to compare the experimental complexes for HPr and its IIA proteins with those predicted for CPr14 (or NPr) and the IIA proteins (Fig. 2). It is noteworthy that CPr14, despite being more similar in sequence to NPr, formed a predicted complex closely resembling those of HPr (Fig. 2).

Although the results for mannitol are not as expected (perhaps NPr interacts with the EIIA^{mtl} but does not transfer its phosphate), the calculated ΔG binding values (in “Hex units”) show that HPr and CPr14 interact with both EIIA^{Glc} and EIIA^{Man}, whereas NPr interacts with neither. Moreover, the HPr-EIIA^{Glc} interaction is stronger than that of CPr14-EIIA^{Glc},

whereas HPr-EIIA^{Man} and CPr14-EIIA^{Man} are roughly equivalent as shown by our experimental data.

Behavior of the Phosphotransfer Protein Variants and Specificity versus Promiscuity—2Δ-HPr cells best utilized sugars that are transported by the Enzyme II of the GFL superfamily, whereas the sugars that were better utilized by CPr14 were substrates of II^{Man} of the Man family. These results were unexpected because the targets that were chosen for site-specific mutagenesis were residues thought to participate in the interaction between HPr and IIA^{mtl} (a GFL family protein) (40). Whether these findings apply to other members of the Man family has yet to be determined.

Because HPr and CPr14 were expressed at near saturating levels, the differences in the rates of uptake are likely to be due to differential interactions with the IIA proteins. However, these interactions could influence the conformations of any of the constituents of an Enzyme II complex and thereby influence the V_{max} of the coupled phosphoryl transfer and transport reactions. Because the differential results obtained with HPr versus CPr14 on mannose/glucosamine/fructose growth and transport are not likely to be due to a differential interaction with Enzyme I, it follows that phosphoryl transfer from HPr to IIA^{Man} must be a rate-limiting step. In this respect, it is interesting to note that sugar transport can be uncoupled from sugar phosphorylation by various mechanisms (41, 42). *In vitro* sugar phosphorylation assays using variable amounts of purified HPr or chimera, saturating amounts of Enzyme I, and limiting amounts of Enzyme II proteins should prove illuminating.

An unexpected finding resulted from growth studies with fructose (Fig. 3 and Table 1) where CPr14 vastly outperformed HPr. Fructose was taken up by the Man EII complex, but the activity of this enzyme for fructose is low (13). We hypothesize that when a sugar appears in the cytoplasm slowly, phosphatases may have more time to hydrolyze a larger fraction of the cytoplasmic sugar phosphate than when the sugar accumulates more rapidly. Thus, we propose a mechanism involving competition for the sugar phosphate substrate by the glycolytic process and intracellular phosphatases.

CPr6, with only 6 of the N-terminal residue changes, was able to support growth relatively well in media containing any one of the several PTS sugars tested (Fig. 4). These few N-terminal mutations in the *npr* gene must have allowed it to fit into the active site of several IIAs and catalyze phosphoryl transfer. However, although these N-terminal residues appear to be critical, they proved not to be sufficient for growth in mannitol, glucitol, or galactitol media. Interestingly, these sugars are all exclusive substrates of their respective Enzyme IIs and are not capable of being transported via II^{Man}. Thus, it appears that these 6 residue changes to NPr are critical for optimal interaction with IIA^{Man}, but they are not sufficient for interaction with the polyol IIAs of the GFL and AG families. It is interesting to note that because these 6 N-terminal residues surround the phosphorylation site in both NPr and HPr, they probably affect catalytic function as well as affinities for their respective Enzyme I and IIA partner proteins.

The presence of selective IIA-HPr function in CPr6 and the strong performance of CPr14 with IIA^{Man} suggest that some C-terminal NPr residues might interact more favorably with

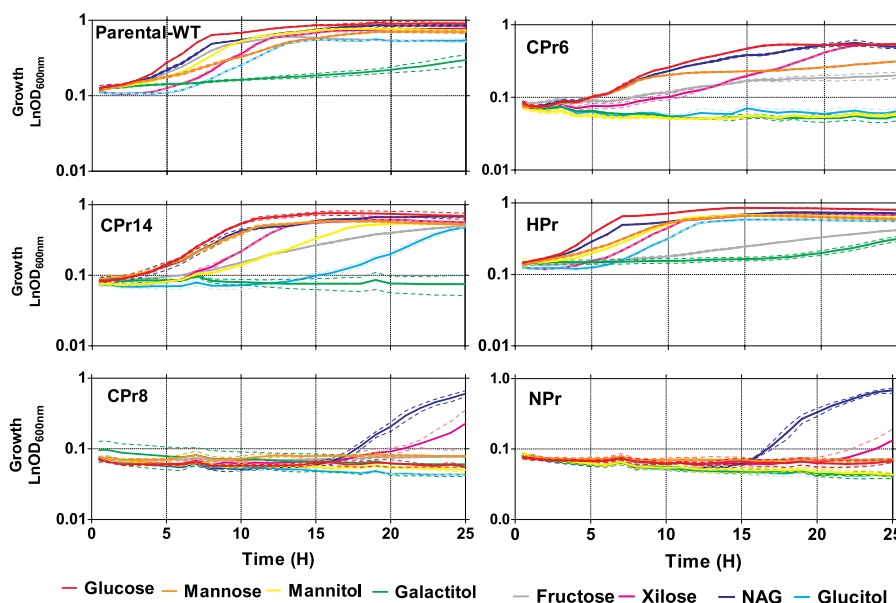


FIGURE 4. Growth of 2Δ with HPr, NPr, CPr14, CPr8, or CPr6 expressed in pZE12. Liquid M9 minimal medium was used with eight different carbon sources at 0.1%, 37 °C. Additionally, 0.2 mM IPTG was uniformly present. The dotted lines indicate S.D. OD, optical density.

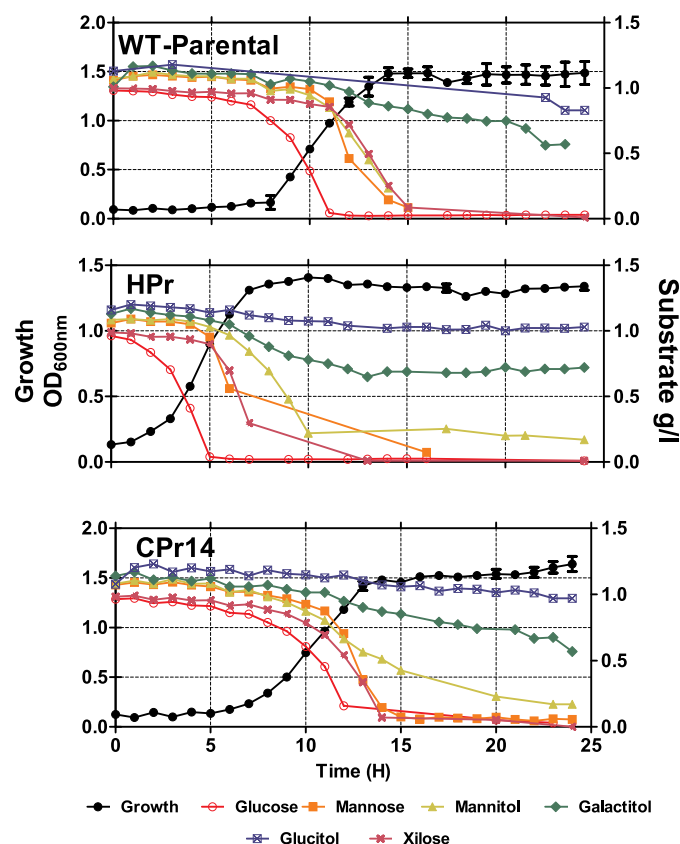


FIGURE 5. Growth and consumption of a mixture of PTS sugars by 2Δ with HPr, NPr, and CPr14. The parental BW25113 (WT) strain is shown for comparative purposes. All strains were grown in triplicate using M9 minimal medium with a mixture of six different sugars as carbon sources (0.1%; 0.2 mM IPTG at 37 °C). OD, optical density.

IIA^{Man} than those of HPr. A possible explanation for this is that HPr may be optimized for phosphoryl transfer to the various IIA proteins *on average* but not for any one protein in particular. Assuming this to be true, our protein engineering therefore

produced a higher performance phosphocarrier protein than HPr, specifically when the phosphoryl acceptor was IIA^{Man}. This possibility had never previously been considered by us or other researchers in the PTS field. The results presented here thus suggest that wild type HPr may not be optimal for phosphoryl transfer to at least several of the IIA proteins.

We have shown that with just a few substitutions, it is possible to convert NPr into a functional HPr. The fact that a mere six point mutations (possibly fewer) in NPr bridge the difference in function between these two proteins highlights the ease with which protein evolution can occur. Computer-assisted analyses shed some light on the possible interactions among the NPr mutants and the targets of HPr. An in-depth understanding of these interactions, however, will require structural studies of the chimeric proteins by NMR and/or x-ray crystallography.

It will be interesting to examine three possible new activities for CPrs. First, potential NPr activity of CPrs should be examined. Due to the subtlety of the *in vivo* NPr mutant phenotype, an *in vitro* NPr activity assay may have to be developed. This might be possible using a phosphorylation assay with purified NPr mutants using IIA^{Ntr} as the substrate. Phosphorylation might also be measured using TrkA, a potassium receptor that binds to IIA^{Ntr} but not to phosphorylated IIA^{Ntr} (43). If CPr has NPr activity, we would have created a dual function protein, a potential snapshot of the protein as an evolutionary intermediate. Second, it should be investigated whether these CPr variants are able to allosterically activate the enzyme glycogen phosphorylase, resulting in the release of individual glucose units akin to HPr in *E. coli* (44, 45). Third, as HPr participates in the co-regulation of expression of the *bglG* operon through phosphorylation of a transcriptional anti-terminator (46), the possibility of some CPr variants to perform the same function should be examined.

Finally, we envisage that the development of NPr mutants able to replace HPr in the transport of PTS sugars may have

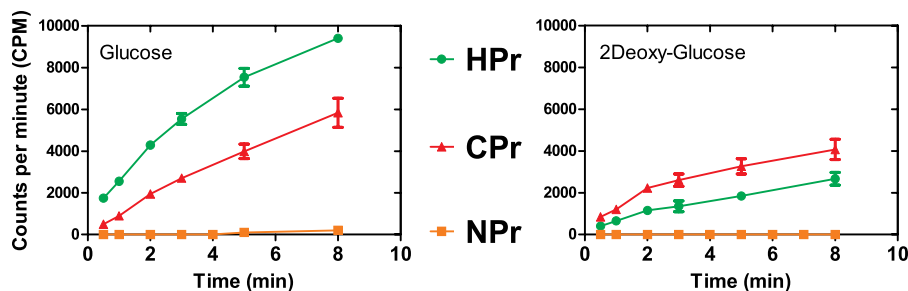


FIGURE 6. Uptake of 2-deoxy-D-[^{14}C]glucose by 2 Δ -HPr and 2 Δ -CPr14 cells. The results represent the average of experiments performed in quadruplicate; S.D. values are indicated by error bars.

biotechnological applications, for example, the simultaneous utilization of mixed sources of PTS and non-PTS sugars or the selective and efficient utilization of specific PTS sugars, whereas leaving others underutilized. Thus, strains expressing different mutant CPrs, each specific for a particular EII, might allow the selective removal/enrichment of specific sugars, a process that could facilitate the production of some sugars for biotechnological purposes as well as the removal of other sugars for bioremediation purposes. It is interesting that nature may have had the same idea; several uncharacterized *E. coli* PTS EII complexes (e.g. Frw and Fry; Ref. 5) have their own EIs and HPrs. The characterization of these self-contained PTS complexes will be relevant to the present studies.

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Genetic Engineering of the Phosphocarrier Protein NPr of the *Escherichia coli* Phosphotransferase System Selectively Improves Sugar Uptake Activity

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