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## Exchange of Phosphoryl Groups between HPr Molecules of the Phosphoenolpyruvate-Dependent Phosphotransferase System Is an Autocatalytic Process<sup>†</sup>

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**ABSTRACT:** HPr, a central component of the phosphoenolpyruvate-dependent phosphotransferase system, can exist in *Escherichia coli* in a phosphorylated (PHPr) and a nonphosphorylated form. We show that, beside the normal transfer of the phosphoryl group from PHPr to enzymes II and III, PHPr can phosphorylate other HPr molecules in an autocatalytic exchange reaction. The reaction is very fast but is inhibited by labeling the protein with Bolton-Hunter reagent. We demonstrate that the exchange reaction can be used to determine the  $\Delta G^\circ$  of the phosphoryl group of mutant forms of PHPr relative to wild-type PHPr. Two HPr mutants were constructed by site-directed mutagenesis, HPr P11E and HPr E68A. Both show altered phosphoryl group potentials but show no significantly altered  $K_M$  or  $V_{max}$  values compared to wild-type HPr, illustrating the sensitivity of the exchange process. The exchange reaction does not occur between HPr from *E. coli* and HPr from *Staphylococcus carnosus*.

The phosphocarrier protein HPr is a central component of almost all bacterial phosphoenolpyruvate (PEP)<sup>1</sup>-dependent sugar phosphotransferase systems (PTS). Its primary function is the catalysis of the transfer of a phosphoryl group from EI, the first general component of the PTS, to the next PTS component, which is sugar-specific (e.g., EIIGlc or EIIMtl; see Figure 1). In *Escherichia coli*, HPr can exist in a phosphorylated form (PHPr) and a nonphosphorylated form. During phosphorylation, the phosphoryl group is carried on the N $\delta$ 1 position of the His15 imidazole ring, as has been reported by several investigators (Weigel et al., 1982a,b; Waygood et al., 1985; van Dijk et al., 1990); the hydrolysis characteristics of this active-site residue have also been es-

tablished (Waygood et al., 1985). In a study on the reversibility of the phosphotransfer reactions catalyzed by HPr, Sutrina et al. (1987) showed that EI, EIIMtl, and EIIGlc can catalyze the exchange of a phosphoryl group between HPr and PHPr.

In the present study, we show that the phosphoryl group exchange between HPr molecules is a highly efficient, autocatalytic process which does not require the presence of EI, EII, or EIII. This self-exchange process enables us to characterize HPr mutants with respect to their phosphoryl group potential.

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<sup>1</sup> Abbreviations: decyl PEG, decyl poly(ethylene glycol) 300; DTT, dithiothreitol; (P)EI, II, III, (phosphorylated) enzyme I, II, III; glc, glucose; mtl, mannitol; PEP, phosphoenolpyruvate; PTS, phosphoenolpyruvate-dependent phosphotransferase system. Note HPr mutants are denoted as follows: HPr AxxB in which A is the wild-type amino acid at residue number xx, which in the mutated HPr is replaced by amino acid B.

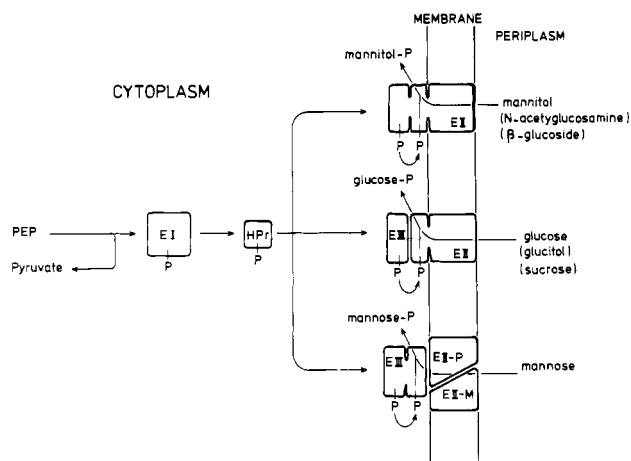


FIGURE 1: Schematic representation of the PTS of several sugars, showing the phosphocarrier protein HPr as a central component.

## MATERIALS AND METHODS

**Materials.** L-[2,5-<sup>3</sup>H]Histidine (55 Ci/mmol), D-[1-<sup>14</sup>C]-mannitol (14 mCi/mmol), and α-D-[U-<sup>14</sup>C]glucopyranoside (140 mCi/mmol) were obtained from Amersham; phosphoenolpyruvate (monocyclohexylammonium salt) and the Bolton-Hunter reagent were obtained from Sigma. The HPLC Mono Q HR5/5 anion-exchange column (ionic capacity 0.27–0.37 mmol/column) and the Q-Sepharose were obtained from Pharmacia. Scintillator 299 was obtained from Packard. Decylpoly(ethylene glycol) 300 was synthesized in our laboratory by B. Kwant.

**Bacterial Strains and Growth.** *E. coli* JC411 (his<sup>-</sup>, arg<sup>-</sup>, met<sup>-</sup>, leu<sup>-</sup>) was transformed with the plasmid pAB65, described by Lee et al. (1982) using standard procedures. Cells were grown on the mineral medium as described by van Dijk et al. (1990). The histidine in the mineral medium had a specific activity of 0.72 mCi/mmol. *E. coli* PB13, containing the plasmid pAB65, was grown on Luria broth, containing 100 μg/mL ampicillin.

**Purification of HPr.** HPr from *E. coli* PB13 was purified as described by van Dijk et al. (1990). For the purification of L-[2,5-<sup>3</sup>H]histidine-labeled HPr from *E. coli* JC411, the Sephadex G75 and the second DEAE column were omitted from the isolation procedure. The selection for HPr-containing fractions on the first DEAE column was more stringent than usual, resulting in a lower yield of the isolation; 6 mg of HPr was isolated from a 1-L culture which is about 60% of the normal yield. The preparation, however, was essentially pure as judged by polyacrylamide gel electrophoresis and isoelectrofocusing and had a specific activity of 14.8 μCi/mg of protein.

**Preparation of *E. coli* Mutant HPr.** The phage M13mp19ptsH was constructed by cloning a 408 bp *Hinc*II/*Sma*I fragment of pAB65 (Lee et al., 1982) containing the whole ptsH gene of *E. coli* into the *Hinc*II site of the M13mp19 polylinker. For site-directed mutagenesis experiments, only the M13 derivatives were used with a reconstituted *Hinc*II site proximal to the M13 phage P<sub>Lac</sub> promoter. All standard DNA manipulations were performed as described elsewhere (Maniatis et al., 1982).

Site-directed mutagenesis was carried out by the phosphorothioate method (Taylor et al., 1985). Mutagenic oligonucleotides were synthesized on an Applied Biosystems DNA synthesizer Model 381 A using the cyanethyl phosphoramidite method (Sinha et al., 1984) and purified as trityl derivatives by HPLC. For the P11E substitution, the 27-mer dGTGCAGACCGTTCTCAGCGGTAATGGT and for the

E68A substitution the 18-mer dCTCGTCTGCGCCTTCTGC were chosen. Finally, mutations were confirmed by dideoxy sequencing (Sanger et al., 1977; Biggin et al., 1983).

**Purification of *E. coli* Mutant HPr.** HPr derivatives were purified from the *E. coli* strain K38 after overexpression using the T7-vector system of Tabor and Richardson (1985). Twenty grams of wet cell paste was suspended in 40 mL of standard buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM DTT, 0.1 mM PMSF, and 0.1 mM EDTA) containing 5 mg of DNase and disrupted by sonification with a Branson sonifier Model B-12. After centrifugation (1 h, 40000g), the supernatant was applied to an ion-exchange column (Q-Sepharose, 3.5 × 10 cm) and eluted with a linear 0.0–0.5 M NaCl gradient in 700 mL of standard buffer. Fractions containing the HPr derivative eluted with 80 mM NaCl in the gradient, whereas the HPr derivative E68A does not bind to the column and could be detected with polyclonal antisera (Dörschug, 1985). HPr-containing fractions were pooled and incubated at 70 °C for 10 min. After precipitation of heat-denatured protein by centrifugation (30 min, 17000g), the clear supernatant was concentrated by pressure dialysis and applied to a gel filtration column (Sephadex G75, 5 × 90 cm). HPr was eluted with 2 L of standard buffer. To obtain pure HPr, however, in some preparations a second ion-exchange column (DE-52, 3 × 8 cm) was necessary, using a 500-mL linear 0.0–0.4 M NaCl gradient in sodium phosphate buffer, pH 6.8. The isolation usually yielded about 100 mg of HPr.

**Purification of *S. carnosus* HPr.** Purification of *S. carnosus* HPr was performed as described elsewhere (Eisermann et al., 1990).

**Preparation of PHPr.** Conditions for the preparation of PHPr were not always the same, but the following conditions are representative. A solution (500 μL) containing HPr (30 μM), EI (0.3 μM), DTT (5 mM), MgCl<sub>2</sub> (5 mM), and PEP (125 μM) in 10 mM Tris-HCl (pH 9.0) was incubated at 30 °C for 10 min to allow complete phosphorylation of HPr. The reaction mixture was then loaded on the Mono Q column. After the A<sub>214</sub> had dropped below 0.03, the separation of PHPr from the other components was performed by using the procedure described below. The PHPr-containing fractions were collected, resulting in a solution that was 5–15 μM in PHPr, depending on the exact isolation conditions. The PHPr solution was immediately put on ice and showed only slow hydrolysis (<10% over 8 h). PHPr was prepared fresh every day.

**Separation of HPr and PHPr.** HPr and PHPr can be separated from each other and EI, PEP, and DTT by HPLC anion-exchange chromatography. A Mono Q anion-exchange column was used with a buffer system consisting of (A) 50 mM Tris-HCl, pH 9.0, and (B) 50 mM Tris-HCl, pH 9.0, plus 500 mM NaCl, using a flow rate of 1 mL/min. Detection was performed by recording the UV absorbance at 214 nm. Gradient conditions started at 5% B in A. Two minutes after sample injection, the percentage of B was raised to 30% in 12 min, during which HPr and PHPr were eluted. After this, the column was rinsed by raising the percentage B to 100% in 3 min. Finally, the column was reequilibrated with 5% B in A. Buffers were usually prepared fresh each day in order to avoid base-line artifacts in the chromatograms.

**Labeling of HPr with the Bolton-Hunter Reagent.** HPr was labeled with the Bolton-Hunter reagent [*N*-succinimidyl 3-(4-hydroxyphenyl)propionate] by adding 75 μL of the reagent (10 mM) in 0.1 M ice-cold borate buffer, pH 8.5, to 75 μL of 0.2 mM HPr in the same buffer. The solution was allowed to stand on ice for 30 min, after which it was frozen at -20 °C until further use.

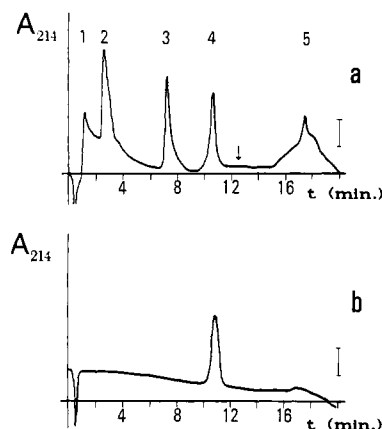


FIGURE 2: (a) Chromatogram showing HPLC Mono Q analysis of a 50- $\mu$ L sample containing 10 mM Tris-HCl (pH 7.6), 5 mM  $MgCl_2$ , 5 mM DTT, 21  $\mu$ M PEP, 3  $\mu$ M EI, and 23  $\mu$ M HPr. The sample was incubated at 30  $^{\circ}C$  for 30 min before it was subjected to the HPLC analysis, which was performed as described under Materials and Methods: 1, DTT and inorganic phosphate; 2, pyruvate; 3, HPr; 4, PHPr; 5, EI. An arrow indicates the elution position of PEP. (b) Chromatogram as in (a) of a 50- $\mu$ L sample of purified PHPr (7  $\mu$ M), prepared as described under Materials and Methods. The vertical bar in both figures is 0.005 absorbance unit.

**Other Enzyme Purifications.** EI was purified as described by Robillard et al. (1979); EII<sup>mtl</sup> was purified as described by Robillard and Blaauw (1987).

**Mannitol Phosphorylation Assay.** PEP-dependent mannitol phosphorylation activities were determined as described by Robillard and Blaauw (1987) in a mixture containing 50 mM potassium phosphate (pH 7.6), PEP (5 mM), DTT (5 mM),  $MgCl_2$  (5 mM), 0.5% Lubrol PX, 0.65  $\mu$ M EI, 4–5  $\mu$ M HPr, 83 nM EII<sup>mtl</sup>, and 10 mM mannitol.

**Determination of Protein Concentrations.** The concentration of HPr was determined by a pyruvate burst, using the procedure described by van Dijk et al. (1990). Concentrations of EI and EII<sup>mtl</sup> were determined by the same procedure, modified with respect to the HPr, EI, and EII<sup>mtl</sup> concentrations as required. The concentration of mutant HPr was calculated from HPLC chromatograms by comparing the 214-nm absorbance of the mutant HPr with that of a wild-type HPr sample of known concentration.

**Other Experimental Procedures.** Polyacrylamide gel electrophoresis was performed according to Laemmli (1970) using 15% acrylamide gels. Isoelectrofocusing was performed as described by van Dijk et al. (1990). Gels were stained by the silver staining procedure described by Wray et al. (1981).

## RESULTS

**Isolation of PHPr, Control of Purity, and Determination of PHPr Concentration.** PHPr can be prepared from HPr, EI, and PEP as described under Materials and Methods. Figure 2a shows the chromatogram of a small-scale PHPr preparation. In order to demonstrate the separation of the components, the reaction mixture was incubated for 30 min at 30  $^{\circ}C$  to allow for the hydrolysis of PHPr after the PEP had been consumed. The normal elution position of PEP is indicated with an arrow. The incubation was carried out at pH 7.6 where hydrolysis is known to be fast compared to hydrolysis at pH 9.0 (Waygood et al., 1985), where most further experiments were performed. It is clear that PHPr can be separated from all other components necessary for HPr phosphorylation.

The PHPr that was isolated by collecting the PHPr-containing fractions was checked for purity in two ways. First

Table I: Elution Positions of Several Forms of HPr during HPLC Analysis with a Mono Q Anion-Exchange Column Using a NaCl Gradient in Tris-HCl Buffer, as Stated under Materials and Methods

HPr form	nonphosphorylated [NaCl] (mM)	phosphorylated [NaCl] (mM)
wild type	85	120
P11E	110	150
E68A	35	100

it was rechromatographed on the Mono Q column using the normal gradient for the separation of HPr and PHPr. Up to 100  $\mu$ L of PHPr solution could be reinjected without prior dilution, even though the solution contained 120 mM NaCl. Larger samples were diluted with 50 mM Tris-HCl (pH 9.0) to prevent direct wash through of PHPr. The absence of any peaks in the chromatogram other than that of PHPr demonstrated the absence of EI or HPr. Figure 2b shows the chromatogram of purified PHPr. The PHPr concentration can be calculated directly from the chromatogram by comparison with a chromatogram of an HPr solution of known concentration. The second control was to assay for PEP-dependent mannitol phosphorylation activity as stated under Materials and Methods. No phosphorylation activity was observed when the purified PHPr preparation was used as the source of HPr unless EI was added separately. The lack of phosphorylation activity demonstrated the absence of any EI in the PHPr preparation.

**Characterization of Other Forms of HPr by the Mono Q Separation Procedure.** The conditions described for the separation of HPr and PHPr can also be used to characterize other forms of HPr which differ from the normal HPr in charge composition. For instance, the HPr P11E contains one more negative charge than the wild-type HPr, changing the elution position from 85 mM NaCl to 110 mM NaCl. This is similar to the change in elution position caused by phosphorylation of the wild-type HPr (Table I). On the other hand, the HPr E68A elutes at a lower salt concentration, 35 mM NaCl, as expected from the net removal of a negative charge.

Table I summarizes the elution positions of HPr and PHPr during HPLC on the Mono Q anion-exchange column. Both mutant HPr forms could be completely phosphorylated by EI and PEP and showed no detectable hydrolysis during the analysis procedure, as judged from the HPLC chromatograms.

**Exchange of Phosphoryl Groups between HPr Molecules.** The exchange of phosphoryl groups between HPr molecules was followed by using a mixture of HPr and L-[2,5- $^3H$ ]-histidine-labeled HPr. The latter will be referred to as  $^3H$ -HPr. The exchange experiment was usually started with PHPr and nonphosphorylated  $^3H$ -HPr, but reverse experiments starting with  $^3H$ -PHPr and HPr were also performed. The exchange process was started by adding PHPr to the nonphosphorylated protein at 30  $^{\circ}C$ . After 1 min, the sample, was loaded on a Mono Q column, and the normal separation procedure was started. During these experiments, the column effluent was collected in 0.5-mL fractions which were subsequently mixed with 3 mL of scintillation fluid and checked for  $^3H$  activity. Figure 3 shows the result of the exchange experiment performed at pH 9.0 in 50 mM Tris-HCl buffer. Figure 3a is the  $^3H$  profile of  $^3H$ -HPr before exchange; Figure 3b is the profile after incubation of the exchange mixture at 30  $^{\circ}C$  for 1 min. The HPr/PHPr ratio calculated from the  $^3H$  content was 0.90 while the molar ratio of the starting HPr to PHPr was 0.85. This shows that the  $^3H$  label has equilibrated over the HPr and PHPr pools. The difference, 0.85 versus 0.90, is within experimental error of the detection

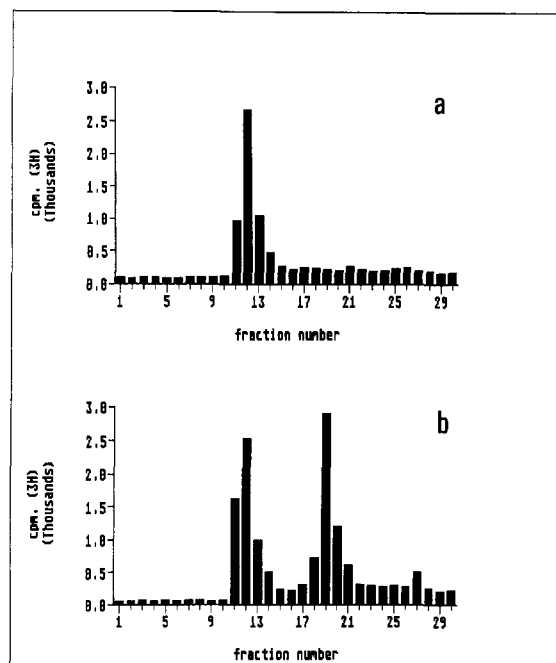


FIGURE 3: (a) Tritium profile of a 30- $\mu$ L sample of  $^3\text{H}$ -HPr (5  $\mu\text{M}$ ) in 50 mM Tris-HCl (pH 9.0) resulting by collecting 0.5-mL fractions during the HPLC Mono Q analysis described under Materials and Methods. The collection of fractions was started 4 min after the start of the analysis. (b) Same analysis as in (a) of a 100- $\mu$ L sample which initially contained  $^3\text{H}$ -HPr (2.5  $\mu\text{M}$ ) and PHPr (3.0  $\mu\text{M}$ ) and 60 mM NaCl in 50 mM Tris-HCl (pH 9.0). The sample was incubated for 1 min at 30  $^{\circ}\text{C}$  before being loaded on the Mono Q column. The sample shows the elution of both HPr and PHPr.

method. It is clear that phosphoryl groups must have been exchanged between the HPr molecules. Since there was no other enzyme component in the exchange mixture besides HPr, we conclude that the exchange process must have been catalyzed by HPr itself.

In order to exclude any possible catalytic activity from minute amounts of EI or even EI fragments, both the PHPr solution and the HPr solution were incubated with polyclonal antibodies against EI (30  $^{\circ}\text{C}$ , 5 min) which are known to completely inactivate 50 nM EI in the PEP-dependent manitol phosphorylation assay. After this, both solutions were filtrated at 4  $^{\circ}\text{C}$  over Centricon filters, having a molecular weight cutoff of 30 K. This treatment did not affect the exchange reaction, indicating that the exchange does not arise from the presence of EI.

Exchange could also be catalyzed by EIII<sup>glc</sup>. However, contamination of the (P)HPr solution with EIII<sup>glc</sup> is very unlikely. EIII<sup>glc</sup> is known to be very well separated from HPr on a DEAE column, as was shown by Weigel et al. (1982a,b). They showed that at pH 7.5 in Tris buffer HPr is eluted from a DEAE column with 50 mM KCl and EIII<sup>glc</sup> with 200 mM KCl. In the isolation procedure for HPr, we also use a DEAE column, operating at almost the same pH [7.6 instead of 7.5, used by Weigel et al. (1982a,b)] and using a 0–100 mM NaCl gradient instead of a KCl gradient. HPr is eluted in our procedure at 30 mM NaCl; EIII<sup>glc</sup> should not elute under the experimental conditions we use. Furthermore, the isolation of purified HPr and PHPr requires additional gel filtration and/or another anion-exchange purification step which should remove any EIII<sup>glc</sup> contamination. In view of these arguments, contamination of (P)HPr solutions with EIII<sup>glc</sup> is highly improbable.

The exchange reaction could be stopped by loading the sample on the Mono Q column. This was checked by loading

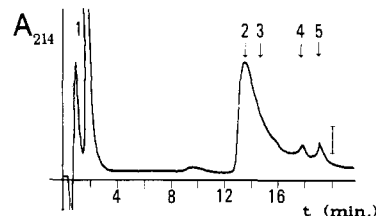


FIGURE 4: HPLC Mono Q chromatogram of a 5- $\mu$ L sample of HPr labeled with Bolton-Hunter reagent as described under Materials and Methods. Prior to analysis, the sample was diluted 10-fold in 50 mM Tris-HCl (pH 9.0): 1, borate and unreacted Bolton-Hunter reagent; 2–5, forms of HPr after labeling with Bolton-Hunter reagent. The elution conditions were described under Materials and Methods. The vertical bar is 0.005 absorbance unit.

PHPr on the column followed by  $^3\text{H}$ -HPr. The  $^3\text{H}$  elution profile did not show the formation of  $^3\text{H}$ -PHPr, indicating that no exchange of phosphoryl groups took place. The Mono Q column was thus used to stop the exchange process when desired.

Various conditions were examined in order to decrease the exchange rates. At lower concentrations of HPr and PHPr, 0.6  $\mu\text{M}$ , equilibrium was still reached within 1 min. The lower concentration limit was set by the sensitivity required to measure the  $^3\text{H}$  profile without going to sample volumes above 200  $\mu\text{L}$ . Increasing the pH had no measurable effect. The exchange was performed at pH 7.6 or 9.0 in 50 mM Tris-HCl and at pH 9.9, 10.6, and 11.5 in 50 mM Tris-glycine. In all cases, equilibrium was reached within 1 min at 30  $^{\circ}\text{C}$ . Equilibrium was also achieved within 1 min when the ratio of HPr/PHPr was varied from 4.0 to 0.3 at pH 9.0 in 50 mM Tris-HCl. Lowering of the temperature to 20  $^{\circ}\text{C}$  did not prevent the equilibrium from being established in 1 min. The rapidity with which equilibrium was established under all conditions prevented us from following the time course of the exchange process.

**Effect of Bolton-Hunter Reagent on Phosphoryl Group Exchange.** Sutrina et al. (1987) also studied the exchange of phosphoryl groups between HPr molecules. Instead of  $^3\text{H}$ -HPr, they used HPr labeled with  $^{125}\text{I}$ -iodinated Bolton-Hunter reagent as a tracer. They concluded that the exchange process was not autocatalytic but that it could be catalyzed by EI, EII<sup>mtl</sup>, or EIII<sup>glc</sup> (Sutrina et al., 1987). The apparent discrepancy with our data could be due to the labeling of HPr with the Bolton-Hunter reagent, which might disturb the interactions between HPr molecules necessary for the exchange process. To examine this, we prepared HPr labeled with the reagent. Figure 4 shows a Mono Q HPLC chromatogram of the resulting HPr preparation. The labeling does not result in a homogeneous preparation; at least four forms of labeled HPr are observed, all at elution positions which indicate a net increase of negative charge, analogous to the findings of Sutrina et al. (1987). The two main fractions, indicated 2 and 3 in Figure 4, were pooled. Mono Q HPLC analysis showed that they could be phosphorylated by EI and PEP. The Bolton-Hunter-labeled HPr was used in the exchange reaction with  $^3\text{H}$ -PHPr. In an analogous experiment, the phosphorylated Bolton-Hunter-labeled HPr was used with  $^3\text{H}$ -HPr. The  $^3\text{H}$  profiles of both experiments are shown in Figure 5a,b. Some  $^3\text{H}$ -HPr was generated from  $^3\text{H}$ -PHPr in the presence of Bolton-Hunter-labeled HPr (Figure 5a), but there was no  $^3\text{H}$ -PHPr generated from phosphorylated Bolton-Hunter-labeled HPr (Figure 5b). The experiment was repeated with higher concentrations of Bolton-Hunter-labeled HPr and PHPr (57 and 6.3  $\mu\text{M}$ , respectively) with a 30-min incubation period instead of the normal 1-min equilibration period. No

Table II: Kinetic Parameters of  $EII^{mtl}$  for HPr in the PEP-Dependent Mannitol Phosphorylation Assay, Described under Materials and Methods<sup>a</sup>

HPr species	$K_M$ ( $\mu$ M)	$V_{max}$ [nmol of mtl $\text{min}^{-1}$ , (pmol of $EII^{mtl}$ ) <sup>-1</sup> ]
wild type	33 ( $\pm 3$ )	3.8 ( $\pm 0.3$ )
HPr P11E	31 ( $\pm 4$ )	3.5 ( $\pm 0.4$ )
HPr E68A	29 ( $\pm 4$ )	3.3 ( $\pm 0.4$ )

<sup>a</sup> Assay conditions in this particular case were 50 mM Tris-HCl (pH 7.6), 20 mM NaF, 10 mM  $\text{MgCl}_2$ , 10 mM PEP, 4 mM DTT, 0.2% decyl PEG, 0.6  $\mu$ M EI, 64 nM  $EII^{mtl}$ , and 1.4 mM mtl. HPr concentrations varied between 2 and 50  $\mu$ M.

phosphorylated Bolton-Hunter-labeled HPr could be detected (UV detection at 214 nm), but 70% of the PHPr was dephosphorylated where normally only 5% is dephosphorylated at this pH. These results indicate that the hydrolysis of PHPr is accelerated by the presence of the Bolton-Hunter-labeled HPr but that phosphoryl group exchange does not occur. Apparently, labeling with the Bolton-Hunter reagent disturbs the interaction between HPr molecules, leading to inhibition of the phosphoryl group self-exchange. The fact that Bolton-Hunter-labeled HPr could still be phosphorylated by EI and PEP but that it could not catalyze self-exchange is in agreement with our data as well as the findings of Sutrina et al. (1987). This will be treated in more detail under Discussion.

**Kinetic Characterization of HPr P11E and HPr E68A.** The HPr P11E and HPr E68A were characterized with respect to their  $K_M$  and  $V_{max}$  on  $EII^{mtl}$  in the PEP-dependent mannitol phosphorylation reaction. The values determined are presented in Table II; reaction conditions are given in the table legend. It is clear from these data that the mutations introduced into the HPr molecule do not significantly affect the kinetic parameters. The data suggest that the mutations do not cause significant changes in the overall structure of the HPr molecule.

**Phosphoryl Group Exchange between HPr and Mutated Forms of HPr or HPr from *S. carnosus*.** The exchange reaction was also performed using wild-type HPr together with mutated forms of HPr, HPr P11E, and HPr E68A as well as with HPr from *S. carnosus*. The exchange process was allowed to occur for 1 min at 30 °C after which the normal Mono Q analysis was performed. The amount of PHPr formed from the mutant HPr's and wild-type PHPr could be directly determined from peak areas since the components were well

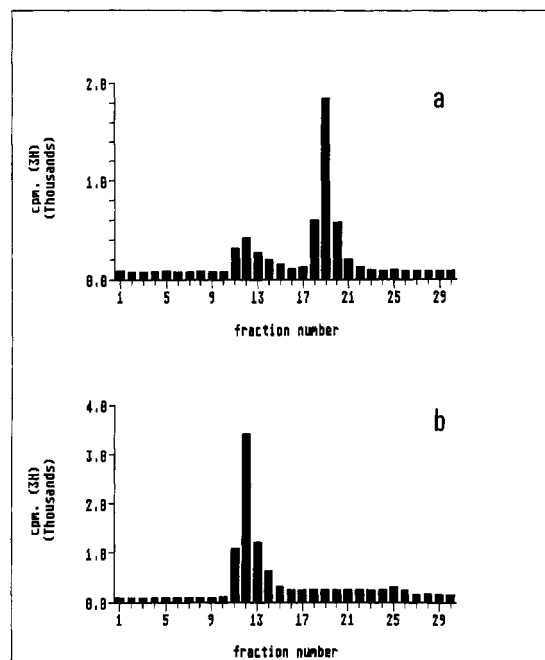


FIGURE 5: (a) Tritium elution profile of HPLC Mono Q analysis of a 200- $\mu$ L sample containing  $^3\text{H}$ -HPPr (1.2  $\mu$ M), purified Bolton-Hunter-labeled HPr (2.0  $\mu$ M), and 60 mM NaCl in 50 mM Tris-HCl (pH 9.0) after 1-min incubation at 30 °C. (b) Same analysis as in (a) of a sample containing  $^3\text{H}$ -HPPr (2.5  $\mu$ M), Bolton-Hunter-labeled PHPr (1.0  $\mu$ M), and 80 mM NaCl in 50 mM Tris-HCl (pH 9.0) after 1-min incubation at 30 °C. Further experimental details are described under Materials and Methods and in the text.

separated by the HPLC separation procedure (see Table I). The situation before and after 1-min exchange at 30 °C is shown in Table III. It is clear that phosphoryl group exchange between wild-type HPr with other forms of HPr was strongly dependent on the particular HPr used. For HPr P11E, all the mutant protein was phosphorylated within 1 min at the expense of the wild-type PHPr. Apparently the phosphoryl group has a lower potential in PHPr P11E, relative to wild-type PHPr. The equilibrium (eq 1) appears to be completely to the right.



In order to try to move the equilibrium to the side of PHPr, comparatively large amounts of wild-type HPr were added to the exchange mixture [up to 20.5  $\mu$ M wild-type HPr in the presence of 1.7  $\mu$ M HPr P11E and 3.8  $\mu$ M PHPr (wild type)].

Table III: Concentrations ( $\mu$ M) of Phosphorylated and Nonphosphorylated Forms of Components in the Exchange Reaction of *E. coli* HPr (wt) with Mutated Forms of *E. coli* HPr or with HPr from *S. carnosus*<sup>a</sup>

before exchange				after exchange			
wt		other HPr		wt		other HPr	
HPr	PHPr	HPr P11E	PHPr P11E	HPr	PHPr	HPr P11E	PHPr P11E
0.0	6.4	4.5	0.0	4.5	1.9	nd <sup>b</sup>	4.5
before exchange				after exchange			
wt		other HPr		wt		other HPr	
HPr	PHPr	HPr E68A	PHPr E68A	HPr	PHPr	HPr E68A	PHPr E68A
0.0	6.4	6.4	0.0	1.9	4.5	4.5	1.9
before exchange				after exchange			
wt		other HPr		wt		other HPr	
HPr	PHPr	HPr <sub>S. carn.</sub>	PHPr <sub>S. carn.</sub>	HPr	PHPr	HPr <sub>S. carn.</sub>	PHPr <sub>S. carn.</sub>
0.0	1.5	6.0	0.0	nd	1.5	6.0	nd

<sup>a</sup> The exchange mixtures (150–200  $\mu$ L) were incubated at 30 °C for 1 min in 50 mM Tris-HCl (pH 9.0). The exchange was stopped by loading the samples on the Mono Q column. Analysis was performed by using the HPLC procedure described under Materials and Methods. Concentrations of the PHPr components were calculated from peak areas in the chromatograms, using a solution of *E. coli* HPr of known concentration as a standard. <sup>b</sup> nd, not detectable, meaning that the concentration is lower than 0.3  $\mu$ M.

However, still at HPr P11E was phosphorylated at the expense of wild-type PHPr.

The situation is quite different when HPr E68A is used instead of HPr P11E. A concentration of 1.9  $\mu$ M PHPr E68A was found after 1 min (Table II) instead of 3.2  $\mu$ M for an equal distribution of phosphoryl groups over wild-type HPr and HPr E68A. This unequal distribution did not change upon lengthening the incubation period to 30 min, demonstrating that it was not caused by a slow exchange rate but reflected the true equilibrium. These data indicate a higher potential of the phosphoryl group on PHPr E68A relative to wild-type PHPr.

No exchange occurred when *S. carnosus* HPr was used in combination with the *E. coli* PHPr. Whether this is caused by an inability of the HPr molecules to form a proper complex or by a large difference in phosphoryl group potential in favor of the *E. coli* PHPr is not clear. Starting with *S. carnosus* PHPr could solve this problem. If the potential of the phosphoryl group in *E. coli* PHPr were much lower than in *S. carnosus* PHPr, a complete transfer of phosphoryl groups should occur upon incubation of *S. carnosus* PHPr with *E. coli* HPr. *S. carnosus* HPr could be phosphorylated by *E. coli* EI, since it was able to replace *E. coli* HPr in a PEP-dependent mannitol phosphorylation assay described and Materials and Methods. The activity was 20% of that observed with the same amount of *E. coli* HPr (R. van Weeghel, unpublished results). In spite of this, we were unable to isolate *S. carnosus* PHPr after phosphorylation from *E. coli* EI and PEP, using the same isolation procedure that was used for the isolation of *E. coli* PHPr. This means that *S. carnosus* HPr can be phosphorylated by *E. coli* EI but the PHPr formed is probably hydrolyzed at a rate that does not permit its isolation with the HPLC procedure described for *E. coli* PHPr.

## DISCUSSION

HPr mediates the transfer of phosphoryl groups to several enzymes (e.g., enzymes II and III). In this paper, we show that HPr can also catalyze the phosphorylation of other HPr molecules, an autocatalytic process that has not been reported before for any of the PTS components. The reaction proceeds to equilibrium at 30 °C within 1 min apparently independent of the ratio HPr/PHPr, HPr concentration, or pH in the range of 7.6–11.5. Also lowering the temperature to 20 °C had no measurable effect.

The HPr/PHPr exchange reaction has also been studied by Sutrina et al. (1987), who labeled their HPr with  $^{125}$ I-Bolton–Hunter reagent. They reported no autocatalytic exchange of phosphoryl groups between HPr molecules. We have shown that attachment of the labeling reagent to the protein inhibits the exchange. Apparently, the label disturbs the interaction of the HPr molecules necessary for the exchange to occur. Sutrina et al. (1987) showed that the labeling does not prevent the phosphorylation of HPr by PEI and thus also allowed EI to catalyze the exchange reaction. When dealing with the heterogeneity of their Bolton–Hunter-labeled HPr, Sutrina et al. (1987) indicated that the different labeled forms of HPr were not equally good substrates for EI. The more highly labeled form seemed to be a poorer substrate. This finding that the reagent disturbed the interaction of EI with labeled HPr explains the low exchange rates in their experiments. They report times of 10 min or more to reach equilibrium whereas we observe that equilibrium is established within 1 min.

The autocatalytic exchange of phosphoryl groups between HPr molecules can be used to characterize mutant forms of HPr. The equilibrium value gives a quantitative measure of

the potential of the phosphoryl group in the mutant HPr, compared to its potential on wild-type HPr, expressed in terms of  $\Delta G^\circ$ . For HPr E68A,  $\Delta G^\circ$  was calculated to be +4.3 kJ/mol, substituting the data from Table II in eq 2 ( $R$  = gas  $\Delta G^\circ$  =

$$-RT \ln ([\text{HPr}][\text{PHPr E68A}]/[\text{PHPr}][\text{HPr E68A}]) \quad (2)$$

constant,  $T$  = absolute temperature). The positive  $\Delta G^\circ$  means that the phosphoryl group is more stable on wild-type PHPr than on PHPr E68A, indicating that the mutation E68A in some way affects the active center of HPr E68A. For HPr P11E, the equilibrium proceeds completely toward PHPr P11E. The mutation P11E thus also in some way influences the active center of HPr. However, in contrast to the mutation E68A, the mutation P11E causes the phosphoryl group to be more stable on HPr P11E than on wild-type HPr. After 1 min of exchange, only PHPr P11E could be detected by our method. Since the equilibrium seems to be far on the side of PHPr P11E, the difference in phosphoryl group potential cannot be quantified in terms of  $\Delta G^\circ$  as was possible for the HPr E68A mutant. Both HPr P11E and HPr E68A thus show altered phosphoryl group potentials compared to wild-type HPr. In contrast to this, the mutant molecules do not demonstrate any difference in  $K_M$  and  $V_{\max}$  values compared to the wild-type enzyme, as is demonstrated by the data in Table II. This fact demonstrates that the exchange process can be used as an additional tool to specifically characterize mutant HPr molecules.

No formation of *S. carnosus* PHPr was observed upon incubation with *E. coli* PHPr. Two factors can be the cause of this. First, the interaction of the two HPr forms could be disturbed, preventing the exchange reaction from occurring. Even though the HPr from *E. coli* and *S. carnosus* show 30% homology, the proper interaction is not guaranteed (Hengstenberg et al., 1989). Second, the potential of the phosphoryl group in *S. carnosus* PHPr may be too high, making the transfer energetically unfavorable. As explained under Results, we were not able to determine which of these explanations was relevant.

The results, presented in this paper, show that the phosphoryl group exchange between HPr molecules is autocatalytic. The process can be used to characterize mutant HPr molecules with respect to the potential of the phosphoryl group. Considering the ease and speed of the analysis, it should be of use in characterizing mutant forms of HPr.

The stereochemical course of reactions leading to the phosphorylation of methyl  $\alpha$ -D-glucopyranoside catalyzed by EII<sup>glc</sup> and to the phosphorylation of mannitol catalyzed by EII<sup>mtl</sup> has been determined by the laboratories of Jacobson and Knowles in an elegant series of experiments (Begley et al., 1982; Mueller et al., 1990). They determined the retention/inversion of configuration at the phosphorus nucleus of the phosphoryl group in the overall process of transfer of this group from PEP to the carbohydrate, starting from chiral [(R)- $^{16}\text{O}$ ,  $^{17}\text{O}$ ,  $^{18}\text{O}$ ]PEP. Since every phosphoryl transfer occurs with inversion of phosphorus configuration, stereochemical analysis of the phosphorylated carbohydrate can tell whether there are an even or an odd number of phosphoenzyme intermediates in the reaction pathway. This approach relies on the assumption that exchange reactions such as observed between HPr and PHPr do not occur under the experimental conditions used. In their experiments, Jacobson, Knowles, and co-workers began with chiral 3-phosphoglycerate (1 mM) which they converted in situ to chiral PEP using enough phosphoglycerate mutase and enolase to perform the task in a few minutes. Extracts of cells were used as the source of

soluble PTS enzymes, EI, HPr and EII<sup>glc</sup>. Waygood et al. (1979) showed that cytoplasmic HPr concentrations in a normal *E. coli* strain are 20–100  $\mu$ M. Furthermore, Mattoo and Waygood (1983) showed that the molar ratio of HPr:EI in such cells is about 10:1, which assures a complete and constant phosphorylation of HPr by EI in the absence of other PTS components, provided enough PEP is present. From the data presented by Begley et al. (1982) and Mueller et al. (1990) and using the numbers given above for cellular HPr concentrations, we estimate the HPr concentration in their experiments to be in the micromolar range. Assuming a turnover number of 250 min<sup>-1</sup> for EII<sup>mtl</sup>, the EII<sup>mtl</sup> concentration in the phosphorylation experiment described by Mueller et al. (1990) can be calculated to be about 30 nM. Since the concentration of PEP is saturating (1 mM), there will be no significant amount of HPr present at any time during the experiment. The situation is the same for the experiment described by Begley et al. (1982). These calculations show that there is no significant pool of unphosphorylated HPr in the experiments described by Begley et al. (1982) and Mueller et al. (1990). Consequently, the phosphoryl group exchange process did not occur and could not compromise their experiments.

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