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# *Escherichia coli* Phosphoenolpyruvate-Dependent Phosphotransferase System: Mechanism of Phosphoryl-Group Transfer from Phosphoenolpyruvate to HPr†

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ABSTRACT: The mechanism of phosphoryl-group transfer from phosphoenolpyruvate (PEP) to HPr, catalyzed by enzyme I of the *Escherichia coli* PEP-dependent phosphotransferase system, has been studied in vitro. Steady-state kinetics and isotope exchange measurements revealed that this reaction cannot be described by a classical ping-pong mechanism although phosphoenzyme I acts as an intermediate. The kinetic data indicate that HPr and PHPr occupy binding sites on enzyme I that do not overlap with the binding sites for PEP and pyruvate. As a result, binding interactions between HPr and enzyme I exist regardless of their phosphorylated state. A general mechanism is presented that describes the phosphorylation of HPr. The physiological implications of this mechanism are discussed.

The *Escherichia coli* phosphoenolpyruvate-dependent phosphotransferase system is responsible for the concomitant translocation and phosphorylation of several sugars across the cytoplasmic membrane (Roseman, 1969; Postma & Roseman, 1976; Saier, 1977; Hays, 1978). The transport process can be described by a minimum of two enzyme-catalyzed reactions:

$$\text{phosphoenolpyruvate} + \text{HPr} \overset{\text{enzyme I}}{\underset{Mg^{2+}}{\leftrightarrow}} \text{PHPr} + \text{pyruvate} \quad \text{(I)}$$

$$\text{sugar}_{out} + \text{PHPr} \overset{\text{enzyme II complex}}{\underset{Mg^{2+}}{\leftrightarrow}} \text{HPr} + \text{sugar-P}_{in} \quad \text{(II)}$$

Sugar phosphorylation and translocation is mediated by several sugar-specific membrane-bound enzyme II complexes, which use PHPr as a phosphoryl-group donor (reaction II). HPr itself is phosphorylated by enzyme I (reaction I).

Since HPr and enzyme I can be purified to homogeneity (Anderson et al., 1971; Dooijewaard et al., 1979; Robillard et al., 1979; Waygood et al., 1980), detailed studies on the initial reactions in the process of phosphorylation and transport are possible. We have previously demonstrated that the active enzyme I molecule is a dimer, which, at low concentrations, dissociates into inactivate monomers (Misset et al., 1980). $Mg^{2+}$ and $Mn^{2+}$ influence the stability and activity of the dimer (Hoving et al., 1982). Most of the accumulated data support a ping-pong mechanism in which HPr only reacts with phosphoenzyme I, as described in Scheme I.

In order to obtain a more complete picture of the molecular interactions between $E_I$ and HPr, we measured the phosphorylation of HPr as well as the isotope exchange between PEP[1] and pyruvate as a function of the concentrations of enzyme I, PEP, and (P)HPr. The results obtained indicate that the phosphoryl-group transfer is not properly described by the mechanism in Scheme I. Apart from the interaction of HPr with $E_I{}^dP$ (reaction Ib in Scheme I), HPr also binds to other enzyme I intermediates such as $E_I{}^d$, $E_I{}^d$–PEP, and $E_I{}^dP$–PYR to form functional complexes. The binding of HPr to $E_I{}^d$ has been confirmed with gel filtration studies of enzyme I. Furthermore, PHPr binds to enzyme I forming a complex that can still react with PEP and pyruvate. From the kinetic data we have concluded that HPr and PHPr occupy binding sites on enzyme I that do not overlap with the binding sites for PEP and pyruvate. The observation that both proteins ($E_I$ and HPr) bind to each other regardless of their phosphorylated state is discussed in terms of a multiprotein PTS complex.

Scheme I

$$2E_I{}^m \underset{k_{-a}}{\overset{k_a}{\rightleftharpoons}} E_I{}^d$$

$$E_I{}^d \underset{\rho_{-1}}{\overset{\rho_1,\ PEP}{\rightleftharpoons}} E_I{}^d\text{-PEP} \underset{r_2}{\overset{\rho_2}{\rightleftharpoons}} E_I{}^dP\text{-PYP} \underset{r_{1,\ PYR}}{\overset{r_{-1}}{\rightleftharpoons}} E_I{}^dP \quad \text{(Ia)}$$

$$E_I{}^dP \underset{\rho_{-3}}{\overset{\rho_{3,\ HPr}}{\rightleftharpoons}} E_I{}^dP\text{-HPr} \underset{r_4}{\overset{\rho_4}{\rightleftharpoons}} E_I{}^d\text{-PHPr} \underset{r_{3,\ PHPr}}{\overset{r_{-3}}{\rightleftharpoons}} E_I{}^d \quad \text{(Ib)}$$

## Materials and Methods

*Bacteria. E. coli* P650 was grown in a 3000-L fermentor at 32 °C in a medium containing the following components (grams per liter): $(NH_4)_2SO_4 \cdot 7H_2O$, 1.0; $K_2HPO_4$, 10.5; $KH_2PO_4$, 4.5; $MgSO_4 \cdot 7H_2O$, 0.1; glucose, 6.0; casamino acids, 1.0; tryptophan, 0.02; thiamin–HCl, 0.05. After the stationary phase was reached, the cells were harvested and washed with 300 L of 1% KCl, after which they were frozen and stored at −20 °C. The yield was approximately 10 kg (wet weight).

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[1] Abbreviations: PEP, phosphoenolpyruvate; α-MeGlc, methyl α-glucopyranoside; PTS, phosphoenolpyruvate-dependent phosphotransferase system; DTT, dithiothreitol; PYR, pyruvate; EDTA, ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; BSA, bovine serum albumin.

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*Salmonella typhimurium* SB 2950 was grown and harvested as stated previously (Dooijewaard et al., 1979).

*HPr* was purified from *E. coli* P650 according to the procedure of Dooijewaard et al. (1979).

*Enzyme II.* The source of enzyme II was the cytoplasmic membrane fraction of *S. typhimurium* SB 2950, which was isolated as described previously (Misset et al., 1980) with 25 mM sodium phosphate buffer, pH 7.0, instead of Tris-HCl buffer.

*Enzyme I* was purified from *E. coli* P650 by the method of Robillard et al. (1979) with the modification as described by Brouwer et al. (1982). Apart from the reason mentioned by Brouwer, this modification was required because $E_I$, isolated solely by hydrophobic interaction chromatography, appeared to be contaminated with nucleic acids (Misset et al., 1980). UV absorption spectra of $E_I$ revealed an absorbance at 260 nm that was much higher than the absorbance at 280 nm. Treatment of $E_I$ with DNase and RNase, followed by several washing steps in an Amicon ultrafiltration apparatus with an UM-20 filter, changed the UV spectrum in that the maximum absorbance appeared at 277 nm and the minimum absorbance at 252 nm. In the modified procedure, $E_I$ eluted from the DEAE-cellulose column (DE-52, Whatman) at 0.23 ± 0.02 M NaCl, after which the pool was concentrated and washed several times on an UM-20 filter. Averaging data from 12 isolation procedures gave the following results. The yield of enzyme I was 12.0 ± 4.0 mg out of 50 g of cells (wet weight), equivalent to 0.25 ± 0.06% of the protein present in the 48000*g* supernatant of the French pressure lysate [as determined by the method of Lowry et al. (1951) using BSA as a standard]. The recovery of the enzymatic activity was 22 ± 7%, resulting in a purification factor of approximately 100.[2] The ratio of the absorbances at 277 and 252 nm was 1.53 ± 0.20. Molar concentrations of enzyme I, determined with [$^{14}$C]PEP (see below), gave a molar extinction coefficient $\epsilon_{280nm}$ = 48000 ± 4000 $M^{-1}$ $cm^{-1}$ equivalent to $\epsilon_{280nm}^{10mg/mL}$ = 3.6 [calculated with a dimer molecular weight of 134000 (Misset et al., 1980; Waygood & Steeves, 1980)]. This experimental value agrees well with the extinction coefficient calculated by Waygood from the amino acid composition of enzyme I: $\epsilon_{280nm}^{10mg/mL}$ = 4.4 (Waygood et al., 1980). When stored at high concentrations (>1 mg/mL), in 25 mM sodium phosphate buffer, pH 7.0, and 1 mM DTT at −20 °C, enzyme I is stable for several months without significant loss of activity.

*Protein* was determined according to Lowry et al. (1951) or by the biuret method, using BSA as a standard.

*Concentrations* of active enzyme I and HPr were determined by measuring the initial burst of pyruvate formation after exposing enzyme I or enzyme I plus HPr to [$^{14}$C]PEP, as described by Brouwer et al. (1982). The result of this method, combined with protein determination of enzyme I (with the biuret method), revealed 0.8–0.9 phosphorylation site/enzyme I dimer.

*Assay Procedures.* The phosphorylation of HPr was measured by following the phosphorylation of methyl α-glucopyranoside or 2-deoxyglucose in the presence of excess concentrations of enzyme II.[3] The results presented in Figures 1 and 2 were obtained from three experiments in which the rate of phosphorylation was measured at varying concentrations of enzyme I and HPr, keeping the PEP concentration constant. After all components except enzyme I and PEP were equilibrated at 37 °C, the indicated amounts of enzyme I were pipetted into the reaction vessels. Subsequently, the time curves were started by addition of the stated concentration of PEP. All three experiments were performed with one stock solution of enzyme I (35 μM), which was kept at 0 °C. Enzyme I was diluted 300-fold in cold buffer shortly before the experiment started. Phosphorylated sugar was separated from the nonphosphorylated sugar and counted as described previously (Misset et al., 1980). Isotope exchange between PEP and pyruvate was measured according to Hoving et al. (1981). All experiments were carried out at pH 7.0 since at this value enzyme I showed maximal activity (data not shown).

*Phosphoenolpyruvate (monopotassium salt) and dithiothreitol* were obtained from Sigma Chemical Co.

*Radioactive-Labeled Compounds.* [$^{14}$C]PEP and methyl α-glucopyranoside and tritiated 2-deoxyglucose were purchased from the Radiochemical Centre, Amersham.

*Sephacryl S-200 and Sephadex G-75* were purchased from Pharmacia Fine Chemicals. All other chemicals were reagent grade.

## Results

*Theory.* The dependence of the steady-state rate of product formation ($V$) on the substrate concentration ($S$) is usually visualized in a Lineweaver–Burk plot in which $V^{-1}$ is set out against $S^{-1}$. In a reaction with two substrates ($S_1$ and $S_2$), $V^{-1}$ is set out against $S_1^{-1}$ at fixed concentrations of $S_2$ and vice versa. The observed patterns are indicative of the reaction mechanism involved. A phosphoryl-group transfer reaction (e.g., the enzyme I catalyzed reaction) is said to operate according to a ping-pong mechanism if the phosphorylated enzyme acts as an obligatory intermediate. The phosphoryl-group donating substrate then reacts only with the unphosphorylated enzyme while the phosphoryl-group accepting substrate only reacts with the phosphorylated enzyme (see, for instance, reactions Ia and Ib in Scheme I). In this case, the Lineweaver–Burk plots show patterns of parallel lines. Furthermore, since the steady-state rate is linearly dependent on the enzyme concentration at all substrate concentrations, the Lineweaver–Burk plot can be recorded at a fixed enzyme concentration. In the case of the enzyme I catalyzed phosphorylation of HPr, the existence of the equilibrium between inactive monomers and active dimers of enzyme I (Scheme I) causes the rate of phosphorylation of HPr to be linear with the total dimer concentration $\sum[E_I^d]$ (=$[E_I^d]$ + $[E_I^d$–PEP] + $[E_I^d$P–PYR] + $[E_I^d$P] + $[E_I^d$P–HPr] + $[E_I^d$–PHPr]) in-

[2] This purification factor differs from the one published by Robillard et al. (1979) (~840) because the bacterium used here (*E. coli* P650) possesses more copies of the genes coding for $E_I$ and HPr. This results in a specific activity of the crude cell extract that is 4 times higher than that found for *E. coli* K235 used by Robillard et al. Therefore a purification factor of 210 should be attainable. The remaining difference of a factor 2 can be attributed to the fact that $E_I$ purified according to the modified procedure reveals a specific activity that is almost half the value published by Robillard [100 vs. 235 μmol of sugar-P $min^{-1}$ (mg of protein)$^{-1}$]. It should be realized that the specific activity is determined in a complementary assay system in which the rest of the necessary PTS components are provided by a crude cell extract of a leaky enzyme I$^-$ mutant. It is possible that some of the variability is caused by fluctuations in the concentration or activity of the PTS components in this extract. Furthermore, the activity determined by this procedure is the rate of sugar phosphorylation, which involves the phosphorylation of enzyme I by PEP and the dephosphorylation by HPr. In the preceding article the specific activity is measured as the exchange activity, i.e., the phosphorylation of enzyme I by PEP and the dephosphorylation by pyruvate. The specific activities as determined by these different procedures refer to different processes, and therefore, neither the values themselves nor the changes in these values should be compared.

[3] As described previously, the phosphorylation of the sugar exhibited a lag time before attaining a steady-state rate (Misset et al., 1980). For our present considerations we only determined the steady-state rates from the time curves.

stead of with the total enzyme I concentration $[E_I]$ ($=\sum[E_I^d] + (1/2)[E_I^m]$) (Misset et al., 1980).

As a result of this monomer–dimer equilibrium, $V$ increases more than proportionally with $[E_I]$ and recording a Lineweaver–Burk plot at a fixed enzyme I concentration will give sets of nonlinear, nonparallel lines. This property of enzyme I makes the Lineweaver–Burk plot meaningless. Proper analysis requires measuring at several $E_I$ concentrations and extrapolating the results to infinite concentration where enzyme I is completely dimerized. This extrapolation can be done in a plot setting out $[E_I]/V$ vs. $V^{-1/2}$ as has been used by Hoving et al. (1981). The dependence of the steady-state rate of phosphorylation ($V$) on the concentrations of $E_I$, PEP, and HPr for the mechanism of Scheme I is given by

$$\frac{[E_I]}{V} = \underbrace{\frac{p_2 + r_2 + r_{-1}}{p_2 r_{-1}} + \frac{p_4 + r_4 + r_{-3}}{p_4 r_{-3}} + \frac{1}{k^{PEP}[PEP]} + \frac{1}{k^{HPr}[HPr]}}_{\text{intercept}} + \underbrace{{}^1/_2\left(\frac{K_D}{k^{PEP}[PEP]}\right)^{1/2}\left(\frac{1}{V}\right)^{1/2}}_{\text{slope}} \quad (1)$$

in which $K_D = k_{-a}/k_a$,[4] $k^{PEP} = p_1p_2r_{-1}/(p_{-1}r_2 + p_{-1}r_{-1} + p_2r_{-1})$, and $k^{HPr} = p_3p_4r_{-3}/(p_{-3}p_4 + p_{-3}r_{-3} + p_4r_{-3})$. $k^{PEP}$ and $k^{HPr}$ are the second-order rate constants of the reactions

$$PEP + E_I^d \xrightarrow{k^{PEP}} E_I^dP + PYR$$

and

$$HPr + E_I^dP \xrightarrow{k^{HPr}} E_I^d + PHPr$$

respectively. In the plot of $[E_I]/V$ vs. $V^{-1/2}$, the intercept (see eq 1) is the value of $[E_I]/V$ where enzyme I is completely dimerized. A plot of these intercepts as a function of the reciprocal substrate concentrations is equivalent to a normal Lineweaver–Burk plot recorded at a single fixed enzyme concentration for a nondissociating enzyme. The validity of a ping-pong mechanism in a system involving a dissociating enzyme, as in Scheme I, can now be checked by testing the substrate dependence of the intercept and the slope of the plot according to eq 1. In order to do this, we must measure the rate of phosphorylation at several enzyme I concentrations for each combination of substrate concentrations.

*Rate Dependence on $E_I$, PEP, and HPr Concentrations.* In order to determine the substrate dependence of the intercept and the slope, the steady-state rate of phosphorylation was measured as a function of the $E_I$, PEP, and HPr concentrations. The rate of phosphorylation was measured at five different enzyme I concentrations for each combination of substrate concentrations, and the results were plotted according to eq 1 (data not shown). If the reaction mechanism of Scheme I is valid, plots of the intercepts vs. the reciprocal PEP or the reciprocal HPr concentration should yield sets of parallel lines. In Figure 1A, the intercepts are plotted against the reciprocal PEP concentration at several fixed HPr concentrations, and in Figure 1B, the same intercepts are plotted

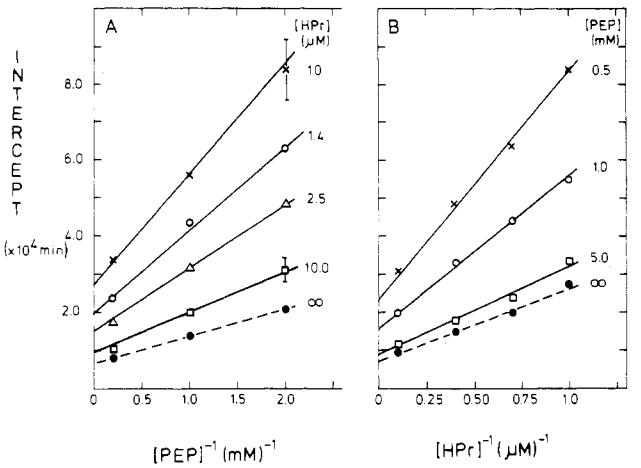



FIGURE 1: Intercepts of the plot $[E_I]/V$ vs. $V^{-1/2}$ (not shown) as a function of reciprocal PEP (A) and HPr (B) concentration. Experimental conditions were as described under Materials and Methods. The rates of phosphorylation were measured at five different enzyme I concentrations (nM): 1.8, 2.5, 3.5, 5.0, and 7.1. The intercept values at infinite HPr concentration [(●) in (A)] were obtained from (B) (and vice versa).

against the reciprocal HPr concentration at several fixed PEP concentrations. Both plots show patterns of nonparallel lines.

Equation 1 predicts that the slopes will be independent of the HPr concentration and vary as the square root of the PEP concentration. In Figure 2A, 2(slope)$^2$ is plotted vs. the reciprocal PEP concentration at different fixed HPr concentrations. The dependence on the PEP concentration is not in accordance with that predicted from eq 1. Furthermore, the slopes are not independent of HPr but decrease with increasing HPr concentrations. Plotting 2(slope)$^2$ vs. the reciprocal HPr concentrations shows a pattern of straight, intersecting lines (Figure 2B). Since the substrate dependence of the intercept and the slope of eq 1 are not in accordance with the experimental results (Figures 1 and 2), we can conclude that the phosphorylation of HPr is not properly described by the reaction mechanism of Scheme I.

*Alterations of Scheme I.* We have tried to alter Scheme I in order to explain the measured substrate dependencies. Competitive substrate inhibition (i.e., formation of the dead-end complexes $E_I^d$–HPr and $E_I^dP$–PEP) can be ruled out as an explanation for the observed kinetics since they are predicted to have no effect on the slope of eq 1 and an effect on the intercepts opposite to that which was experimentally found.

Hydrolysis of the phosphorylated enzyme intermediate at a rate comparable to the overall reaction velocity could be a possible explantation. It would alter the parallel lines pattern of the intercepts into an intersecting lines pattern while the slope of eq 1 would become

$$\text{slope} = \frac{1}{2}\left[\frac{K_D}{k^{PEP}[PEP]}\left(\frac{k_h}{k^{HPr}[HPr]} + 1\right)\right]^{1/2} \quad (2)$$

(compare eq 2 with Figure 2B). $k_h$ is the first-order hydrolysis rate constant of $E_I^dP$. We carried out an experiment in order to establish to what extent hydrolysis of $E_I^dP$ occurred. Parallel experiments, in which either the overall reaction rate was measured via the formation of [$^{14}$C]-$\alpha$-MeGlc-6-P (see Materials and Methods) or the hydrolysis of $E_I^dP$ via the formation of [$^{14}$C]pyruvate, revealed no differences in these formation rates. There should be a difference, however, if a fast hydrolysis of $E_I^dP$ and/or PHPr occurred. Therefore $E_I^dP$ and PHPr do not hydrolyze at a rate comparable with the

[4] $K_D$ denoted here is in fact an apparent $K_D$ since it is dependent upon the $Mg^{2+}$ concentration. Since $Mg^{2+}$ was held constant at 5 mM in all the kinetic experiments presented in this paper, the equilibria between monomers, dimers, and $Mg^{2+}$-complexed dimers of enzyme I can be described by one $K_D$ [see Hoving et al. (1982) and Discussion of this paper].

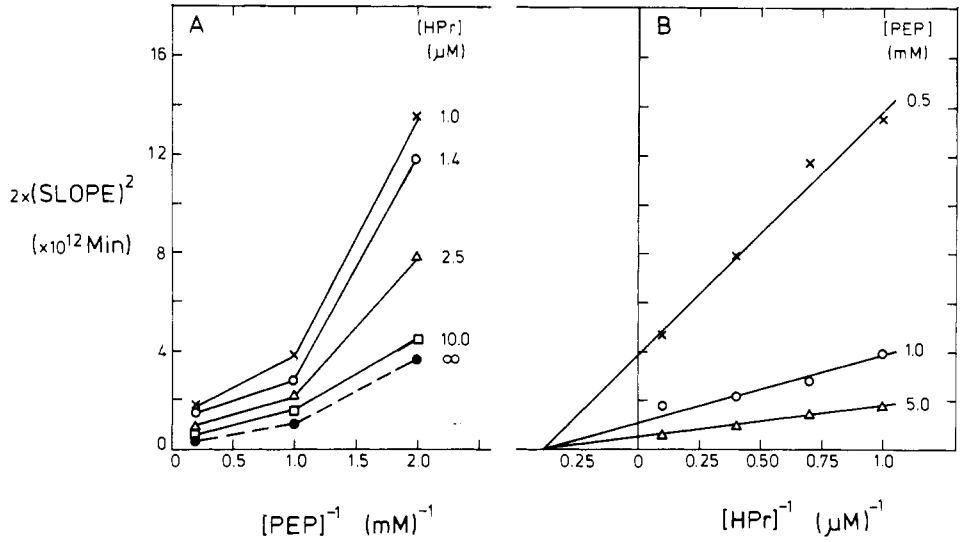



FIGURE 2: Two times the square of the slope of the plot $[E_I]/V$ vs. $V^{-1/2}$ as a function of the reciprocal PEP (A) and HPr (B) concentration.

overall rate of phosphorylation and cannot account for the result in Figures 1 and 2B.

Dissociation of $E_I{}^dP$ cannot explain the observed substrate dependence of the slope and intercept of eq 1. Assuming a $K_D'$ for $E_I{}^dP$ alters the slope of the eq 1 into

$$\text{slope} = \frac{1}{2}\left(\frac{K_D}{k^{PEP}[PEP]} + \frac{2K_D'}{k^{HPr}[HPr]}\right)^{1/2} \quad (3)$$

while the intercept remains unaltered. Neither of these predicted behaviors fits the data in Figure 2A,B.

From the observations that (i) the steady-state kinetics of the enzyme I catalyzed reaction are not in accordance with Scheme I, (ii) this behavior is found over a wide range of PEP and HPr concentrations, and (iii) this behavior cannot be explained by competitive substrate inhibition, hydrolysis of $E_I{}^dP$, or dissociation of $E_I{}^dP$, we must conclude that HPr and PEP form functional complexes with other enzyme I forms in addition to those described in Scheme I. This can be understood by a closer examination of the reaction mechanism of Scheme I.

The prediction of eq 1 that the intercepts, when plotted against the reciprocal substrate concentrations, should yield sets of parallel lines stems from the fact that PEP and HPr each react with a chemically different form of enzyme I ($E_I{}^d$ and $E_I{}^dP$; see Scheme I). Since this ping-pong mechanism does not contain enzyme intermediates that bind PEP (or pyruvate) and HPr (or PHPr) at the same time, no product terms of the PEP and HPr concentrations appear in eq 1. However, the results in Figure 1 clearly show that product terms are present, and therefore, it may be concluded that PEP (or pyruvate) and HPr (or PHPr) form functional ternary complexes with enzyme I. These extra interactions will affect not only the intercept of eq 1 but also the slope. As has been pointed out in the preceding paper (Hoving et al., 1982), the slope is a measure for the dissociation of enzyme I. The ping-pong mechanism of Scheme I resulted in only a PEP dependence of the slope, but since we concluded that ternary complexes of PEP (or pyruvate) and HPr (or PHPr) with enzyme I are also involved, the slope will become dependent on the HPr concentration as well. In the next sections we will demonstrate (i) the direct phosphoryl-group transfer from $E_I{}^dP$ to HPr, (ii) the binding of HPr to $E_I{}^d$, and (iii) the binding of PHPr to $E_I{}^d$ whereby the dimer of enzyme I still can be phosphorylated by PEP.

*Phosphoryl-Group Transfer from $E_I{}^dP$ to HPr.* Incubation of enzyme I with PEP and $Mg^{2+}$ yields phosphorylated enzyme I and pyruvate (see Materials and Methods). Since the equilibrium of this reaction lies far to the pyruvate and $E_I{}^dP$ side, incubation of equal amounts of enzyme I and PEP will give a complete conversion to $E_I{}^dP$ and pyruvate (Hoving et al., 1981, 1982). This was confirmed by Sephadex G-75 gel filtration of such an incubation mixture. Enzyme I was eluted at 0 °C with a buffer containing EDTA (0.5 mM) to prevent hydrolysis of $E_I{}^dP$ [see Hoving et al. (1982)]. The enzyme I was completely phosphorylated as judged by the inability to rephosphorylate it with [$^{14}$C]PEP (see Materials and Methods). In order to determine whether phosphoryl-group transfer from $E_I{}^dP$ to HPr occurs, we incubated stoichiometric amounts of PEP and enzyme I (each 10 μM) for 15 s. Lactic dehydrogenase and NADH were included in the incubation mixture to convert all pyruvate to lactate. Subsequently, HPr was added to a final concentration of 50 μM, and after 15 s, the incubation mixture was cooled to 0 °C and 5 mM EDTA was added. The reaction mixture was loaded on a Sephadex G-75 column and eluted with EDTA-containing buffer (see above). Peaks of enzyme I and HPr activity were well separated. In this case enzyme I could be quantitatively rephosphorylated, indicating that the $E_I{}^dP$ that was formed during the first 15 s of the incubation could transfer its phosphoryl group to HPr in the absence of PEP and pyruvate.

*Binding of HPr to $E_I{}^d$.* We cannot determine from the kinetic data alone whether HPr binds to all enzyme I intermediates present in reaction Ia (i.e., $E_I{}^d$, $E_I{}^d$–PEP, $E_I{}^dP$–PYR, and $E_I{}^dP$). In the previous section we have demonstrated the transfer of the phosphoryl group from $E_I{}^dP$ to HPr, substantiating the existence of an $E_I{}^dP$–HPr complex. Although it is possible to obtain experimental conditions in which the complex $E_I{}^d$–PEP or $E_I{}^dP$–PYR is preferentially present, studies on the binding of HPr to these complexes are difficult to carry out because HPr would be phosphorylated immediately. $E_I{}^d$, however, does exist in the absence of PEP [see Hoving et al. (1982)], enabling us to investigate the binding of HPr to $E_I{}^d$ in the concentration range used in the kinetic experiments (up to 20 μM HPr). This interaction can be visualized with gel filtration chromatography. The ratio of enzyme I dimers to monomers is dependent upon the enzyme concentration (Misset et al., 1980; Hoving et al., 1982). Increasing the $E_I$ concentration increases the dimer/monomer ratio. The weight-averaged molecular weight of $E_I$ therefore

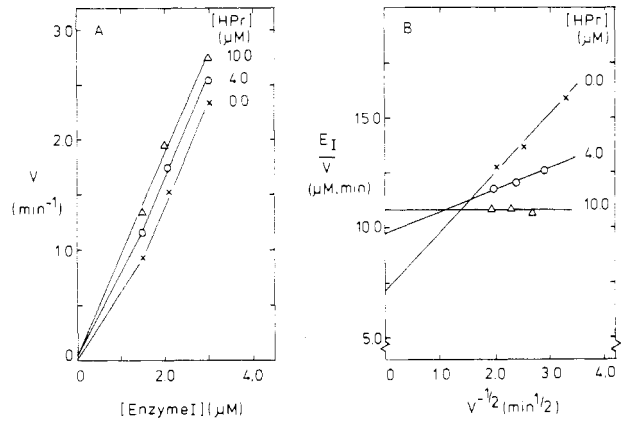



FIGURE 3: (A) Initial rate of enzyme I phosphorylation in the absence and presence of HPr. Experimental conditions: 25 mM sodium phosphate buffer, pH 7.0, 1 mM DTT, 1 mM $NaN_3$, 200 μM EDTA, 60 μM $MnCl_2$, 0.25 μM [$^{14}$C]PEP, and the stated concentrations of enzyme I and HPr. Separation of pyruvate and PEP and determination of the rate of phosphorylation are described in the preceding paper (Hoving et al., 1982). (B) Replot of the data from (A).

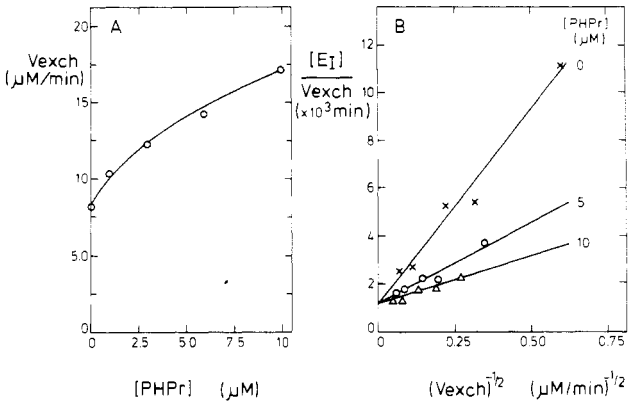


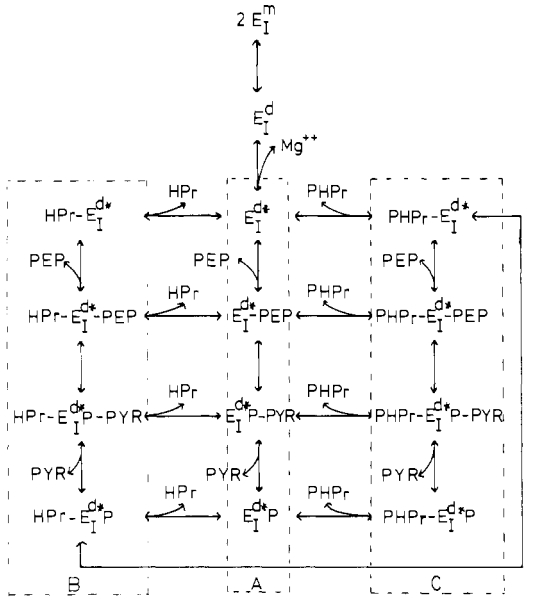

FIGURE 4: (A) Rate of isotope exchange between PEP and pyruvate as a function of [PHPr]. Experimental conditions: 25 mM sodium phosphate buffer, pH 7.0, 2.5 mM $MgCl_2$, 1 mM DTT, 1 mM $NaN_3$, 250 μM PEP, 2.5 mM pyruvate, and 30 nM enzyme I. The experiment was performed as described under Materials and Methods. (B) $[E_I]/V_{exch}$ vs. $V_{exch}^{-1/2}$. The rate of isotope exchange was measured at the indicated concentrations of PHPr at five different enzyme I concentrations (nM): 30, 50, 100, 200, and 400. Experimental conditions are the same as in (A). Qualitatively similar results were obtained with 25 μM PEP and 5.0 mM pyruvate.

varies from 67 000 (monomer) to 135 000 (dimer). This can be determined on a Sephacryl S-200 column as has been done in the preceding paper (Hoving et al., 1982). An agent that binds selectively to either the monomers or the dimers will also alter the ratio. From our kinetic experiments we have concluded that HPr might interact with $E_I^d$. Therefore we have examined the effect of HPr on the elution position of enzyme I on Sephacryl S-200. The interaction of HPr and $E_I^d$ should increase the ratio of dimers/monomers, shifting the elution position of enzyme I toward the dimer position. A sample of enzyme I, eluting at a position with a molecular weight of 87 000, shifts in the presence of 5 and 20 μM HPr to positions with molecular weights of 95 000 and 103 000, respectively. The observed shift of the elution position of $E_I$ toward the dimer position was found only to occur in the presence of $Mg^{2+}$ or $Mn^{2+}$ but not in the absence of these metal ions. This result confirms the interaction of HPr with $E_I^d$ as originally proposed from the kinetic data.

This interaction implies, however, that HPr-complexed enzyme I can be phosphorylated by PEP. In order to compare the specific activity of the uncomplexed dimer of enzyme I with the HPr-complexed dimer, we measured the initial rate of phosphorylation of enzyme I, as described in detail in the preceding paper (Hoving et al., 1982). The rate values obtained equal $k^{PEP}[E_I^d]$ and will be dependent upon the total enzyme I concentration. Due to the monomer–dimer equilibrium of enzyme I, the rate will increase more than proportionally with the total enzyme I concentration. This was found experimentally [Figure 3A, (×)]. Addition of HPr increases the initial rate of enzyme I phosphorylation [Figure 3A, (○) and (△)]. This confirms that HPr-complexed dimers of enzyme I are phosphorylated as well. Whether the increased phosphorylation rate is due to a higher specific activity of $E_I^d$–HPr or solely to a higher total dimer concentration can be established by extrapolating the results of Figure 3A to 100% dimers of enzyme I. This can be done in a plot according to eq 1 [see Hoving et al. (1982)]. Figure 3B shows this plot. From the fact that the intercept (i.e., the reciprocal specific activity of the dimer) slightly increases with HPr, it can be concluded that the enzyme I dimer, when it is complexed with HPr, is phosphorylated at a slightly decreased rate. The HPr-dependent stimulation of the initial rate of phosphorylation (Figure 3A), therefore, can be attributed to the fact that HPr increases the total enzyme I dimer concentration, which is reflected by a decrease of the slope in Figure 3B.

*Binding of PHPr to Enzyme I.* The interaction of PHPr with enzyme I can be studied by measuring the effect of PHPr on the isotope exchange between PEP and pyruvate, a reaction that is catalyzed by enzyme I and has been studied in detail by Hoving et al. (1981, 1982). In contrast with the steady-state kinetics of the phosphorylation of HPr, this reaction is monitored in a situation of chemical equilibrium. Addition of HPr to an isotope-exchange reaction will, in chemical equilibrium, result in an almost complete phosphorylation of HPr, as can be deduced from the experiment described above in which phosphoryl-group transfer from $E_I^dP$ to HPr was established. This enables us to study the interactions of PHPr with enzyme I. The mechanism in Scheme I predicts that addition of PHPr to an isotope-exchange experiment should decrease the rate of exchange since PHPr complexes with $E_I^d$, thus lowering the amount of enzyme I available for the isotope-exchange reaction. However, from the steady-state kinetics of the phosphorylation of HPr, we concluded that PHPr may be part of functional ternary complexes (with PEP and/or pyruvate), and from this we can predict that PHPr will increase the rate of isotope exchange. Figure 4A shows that addition of PHPr increases the rate of isotope exchange when measured at fixed concentrations of $E_I$, PEP, and pyruvate. We must conclude, therefore, that PHPr forms complexes with enzyme I that are still capable of catalyzing isotope exchange between PEP and pyruvate. This confirms the existence of ternary complexes of PHPr and PEP and/or pyruvate with enzyme I. Whether the increased exchange rate is due to a higher specific activity of the $E_I^d$–PHPr complex or solely to a higher total dimer concentration can be established by measuring the rates of isotope exchange as a function of the enzyme I concentration and extrapolating the results to 100% dimers. This can also be done in a plot according to eq 1 [see Hoving et al. (1981)]. Figure 4B shows this plot. From the fact that PHPr hardly affects the intercept we can conclude that the specific activity of the PHPr-complexed dimer is almost the same as the specific activity of the uncomplexed dimer. The stimulation in Figure 4A, therefore, can be attributed mainly to the fact that PHPr—in analogy with HPr—increases the total enzyme I dimer concentration, which is reflected by a decrease of the slope in Figure 4B.

Scheme II




## Discussion

*Proposed Mechanism.* The phosphorylation of HPr, which is catalyzed by enzyme I, cannot be described by the simple ping-pong mechanism presented in Scheme I. This has been concluded from the kinetic experiments described under Results. In order to propose a new mechanism, we will first summarize all the available data that must be included in it. (i) The active form of enzyme I is the dimer that is in equilibrium with its inactive monomers (Misset et al., 1980). (ii) In order to become phosphorylated, the dimer of enzyme I should be complexed with one metal ion [$Mg^{2+}$ or $Mn^{2+}$; see Hoving et al. (1982)]. (iii) The monophosphorylated enzyme I dimer acts as an intermediate in the transfer of the phosphoryl group from PEP to HPr. This is based upon the observations that $E_I{}^dP$ can transfer its phosphoryl group to HPr and that the phosphorylation rate of enzyme I is comparable with the overall phosphorylation rate of the sugar. (iv) The stoichiometry of the reaction is presumably one PEP and one HPr molecule reacting with the enzyme I dimer. This must be concluded from the fact that enzyme I can only be phosphorylated at one site per dimer (Materials and Methods; see discussion below). (v) In order to become phosphorylated, HPr can bind not only to $E_I{}^dP$ but also to other forms of enzyme I that are present during its phosphorylation: $E_I{}^d$, $E_I{}^d$–PEP, and $E_I{}^dP$–PYR. The observed interactions of HPr with $E_I{}^dP$ and of HPr with $E_I{}^d$ (Figure 3) suggest that HPr also interacts with $E_I{}^d$–PEP and $E_I{}^dP$–PYR. (vi) HPr only binds to the metal ion complexed dimer of enzyme I (Figure 3). (vii) The enzyme I dimer can be phosphorylated regardless of whether it is complexed with HPr or PHPr (Figures 3 and 4). (viii) Uncomplexed dimers of enzyme I dissociate more easily than complexed dimers. Complexation of $E_I{}^d$ with $Mg^{2+}$ (Hoving et al., 1982), PEP, HPr, and PHPr results in more stable dimers (Figures 2–4). This is also true for $E_I{}^dP$ as judged from the elution position on Sephacryl S-200 (Misset et al., 1980; Hoving et al., 1982). Combining (i)–(viii) results in the mechanism given in Scheme II. In this mechanism, the (active) metal ion complexed dimer is represented by $E_I{}^{d*}$. Reaction A is the phosphorylation of enzyme I, which can be measured by isotope exchange between PEP and pyruvate in the absence of HPr (Hoving et al., 1981). Reaction B describes the phosphorylation of enzyme I when it is complexed with HPr (Figure 3), while reaction C describes the phosphorylation of enzyme I in the presence of PHPr and explains the observation that isotope exchange between PEP and pyruvate is enhanced in the presence of PHPr (Figure 4). Reaction A is coupled to B and C by the addition of HPr and PHPr, respectively, to the individual enzyme complexes of reaction A. Therefore, HPr and PHPr increase the total dimer concentration that can be phosphorylated by PEP.

The mechanism of Scheme II differs from the one presented by Waygood & Steeves (1980). They concluded that Scheme I is the proper mechanism for the enzyme I catalyzed phosphorylation of HPr. Their conclusion was based on the fact that the Lineweaver–Burk plot (in which $V^{-1}$ is set out against $[PEP]^{-1}$ at fixed HPr concentrations and vice versa) showed a pattern of parallel lines. However, this Lineweaver–Burk plot was recorded at a single, fixed enzyme I concentration, thus neglecting the influence of the monomer–dimer equilibrium of enzyme I. In the theoretical section we have shown that such an approach is incorrect.

*$E_I{}^dP$ Stoichiometry.* The enzyme I dimer can only be phosphorylated at one site. This was concluded from concentration determinations using [$^{14}$C]PEP and measuring the burst of pyruvate formation (see Materials and Methods). The reliability of this method has been checked by showing that the total number of sites found increases linearly with the total enzyme concentration (under the experimental conditions employed, enzyme I is completely dimerized). Furthermore, when applied to determining the concentration of HPr, this same technique gave a value of 0.8–0.9 phosphorylation site/9600 daltons (using the biuret method to determine the protein content). The result with enzyme I raises the question of whether the monomers are identical or not. Purified enzyme I exhibits one band on regular and sodium dodecyl sulfate–polyacrylamide gels, suggesting no large differences in charge or size. These methods, however, do not enable us to detect minor differences in subunit composition that can give rise to only one phosphorylation site. It is possible that one monomer binds PEP and is phosphorylated while the other binds HPr. With our present knowledge of enzyme I it is impossible to say whether it shows half-of-the-sites reactivity as is found for several other oligomeric enzymes (Levitzki & Koshland, 1976) or a flip-flop process. More insight into the nature of the binding sites of PEP and HPr on either the monomers or the dimer of enzyme I is required before such statements can be made.

*The PTS: A Multiprotein Complex?* In the literature, the PTS is considered to be a two-phase system consisting of cytoplasmic (i.e., water-soluble) proteins ($E_I$, HPr, $III^{Glc}$) and the integral membrane-bound (i.e., water-insoluble) proteins (IIA, IIB, $IIB^{Glc}$). This consideration is primarily based on the fact that the soluble proteins are found in high-speed supernatants of cell-free extracts and can be handled in aqueous media without the use of detergents, whereas the membrane-bound proteins demand the use of detergents in order to be extracted from the membrane and kept in solution. The enzyme I catalyzed phosphorylation of HPr is supposed to occur in the cytoplasm. PHPr then diffuses to the membrane in order to be available as substrate for enzyme II in the sugar translocation and phosphorylation reaction. There are, however, several indications that enzyme I and HPr may be associated with the membrane surface, thus allowing the possibility of a protein complex of $E_I$, HPr, and $E_{II}$. (i) Enzyme I is a hydrophobic protein. In our laboratory, the enzyme is purified by using hydrophobic interaction chromatography. It binds so strongly that it can only be removed by lowering the solvent polarity or using detergents. This hydrophobic

character of enzyme I may play a role in its binding to the membrane. (ii) Isolation of the cytoplasmic membrane fraction from a wild-type *E. coli* (i.e., containing $E_I$, HPr, and $E_{II}$) via differential centrifugation yields a membrane pellet containing HPr and $E_I$ in amounts exceeding those expected for a cytoplasmic protein that has no affinity for the membrane (O. Misset, unpublished data). (iii) When a crude cell extract is subjected to sucrose density gradient centrifugation, one expects that cytoplasmic proteins will remain at the top of the gradient and heavier particles, like membrane vesicles, sediment to their equilibrium position. Indeed, $E_{II}$ activity is found at a density of 1.17 g/cm$^3$, which is expected for cytoplasmic membrane vesicles (Osborn et al., 1972). However, although most of the $E_I$ activity remains at the top of the gradient, the peak is very asymmetric and tails into the gradient toward the position of the cytoplasmic membranes. This can be explained by the fact that initially an amount of $E_I$ was bound to the membranes, which dissociates from the membranes during centrifugation through the sucrose gradient (O. Misset, unpublished data). (iv) Right-side-out vesicles, prepared by an osmotic shock treatment that resulted in the release of the soluble proteins, were, when loaded with PEP, able to transport and phosphorylate $\alpha$-MeGlc (Kaback, 1968). Since vesicles, prepared from an enzyme I mutant, failed to accumulate the sugar, Kaback suggested that part of enzyme I and HPr were associated with the cytoplasmic membrane and could not be removed by the osmotic shock treatment. (v) The PTS of *Rhodopseudomonas sphaeroides* consists of an integral membrane-bound protein ($E_{II}$) and a fully membrane-associated enzyme I type protein called Soluble Factor (Saier et al., 1971). Like $E_I$, Soluble Factor can be phosphorylated by PEP. Recently, Brouwer et al. (1982) have demonstrated that the Soluble Factor is a tightly or even covalently linked complex of an $E_I$- and HPr-like molecule. This is consistent with our finding that HPr can bind to unphosphorylated $E_I$ although this interaction is weaker than in the Soluble Factor.

These observations make the existence of a PTS protein complex in *E. coli* plausible. One of the consequences of such a protein complex would be that interactions between the proteins exist, regardless of their phosphorylated state. Scheme II shows that such an interaction exists between (P)HPr and (phospho)enzyme I. More evidence can be obtained from mechanistic studies of the enzyme II catalyzed reaction. Another approach would involve treatment of whole cells with cross-linking agents in order to stabilize the presumed protein complex and prevent it from dissociation during cell rupturing and subsequent analytical procedures. These experiments are now in progress.

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