Transient State Kinetics of Enzyme I of the Phosphoenolpyruvate:Glycose Phosphotransferase System of *Escherichia coli*: Equilibrium and Second-Order Rate Constants for the Phosphotransfer Reactions with Phosphoenolpyruvate and HPr†

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ABSTRACT: The first two reactions in the phosphotransfer sequence of bacterial phosphoenolpyruvate: glycose phosphotransferase systems are the autophosphorylation of Enzyme I by phosphoenolpyruvate followed by the transfer of the phospho group to the low-molecular weight protein, HPr. Transient state kinetic methods were used to estimate the second-order rate constants for both phosphotransfer reactions. These measurements support previous conclusions that only the dimer of Enzyme I, EI_2, is autophosphorylated, and that the rate of formation of dimer is slow compared to the rate of its phosphorylation. The rate constants of the two autophosphorylation reactions of EI_2 by PEP are 6.6×10^6 M^{-1} s^{-1}, and differ from one another by a factor of less than 3. The rate constant for the transfer reaction between phospho-EI_2 and HPr is unusually large for a covalent reaction between two proteins (220×10^6 M^{-1} s^{-1}), while the constant for the reverse reaction is 4.2×10^6 M^{-1} s^{-1}. Using the previously reported equilibrium constant for the autophosphorylation reaction, 1.5, the overall equilibrium constant for phosphotransfer from PEP to HPr is 80, somewhat higher than that previously reported. The results also show that EI_2 can phosphorylate multiple molecules of HPr without dissociating to a monomer (EI), and that EI can accept a phospho group from phospho-HPr. These results are directly applicable to predicting the rates of phosphoenolpyruvate phosphotransferase system sugar uptake in whole cells.

The phosphoenolpyruvate phosphotransferase system (PTS)[1] is a major pathway for the uptake of carbohydrates in eubacteria (*1*, *2*). Sugars that are PTS substrates are phosphorylated as they are translocated across the cell membrane. The PTS also serves several major regulatory functions, including the direct regulation of the uptake of some carbohydrates that are not its own substrates and the indirect regulation of carbon metabolism on a broader scale through strong control of the activity of adenylate cyclase. Although a single bacterial species may contain as many as 20 PTS systems for at least as many sugar substrates, all (but one, fructose-specific) of the systems in a given species use the same two proteins, Enzyme I and HPr, for the initial steps of the pathway: the autophosphorylation of Enzyme I from PEP followed by the transfer of the phospho group to HPr.

Enzyme I is a PEP-driven protein (HPr) kinase, with the following properties. (1) Enzyme I is active only as the homodimer, EI_2, consisting of two 64 kDa monomers (*3*−*9*). (2) The monomer consists of two domains, of approximately equal size, defined by calorimetry (*10*), genetic dissection (*11*−*14*), and limited proteolysis (*10*) experiments. (3) The amino-terminal domain (EI-N) contains the phosphorylation site (His 189) and is the domain that interacts with HPr in the phosphotransfer reaction (*11*, *12*, *14*, *15*). The carboxy-terminal domain (EI-C) contains the PEP binding site and also mediates dimerization (*13*, *16*; H. Patel and K. Vyas, personal communication). (4) The equilibrium constants for the monomer−dimer transition have been determined (*17*, *18*; H. Patel and K. Vyas, personal communication) and are strongly influenced by ligands, including Mg^{2+} and PEP, ionic strength, pH, and temperature (the reaction is endothermic) (*10*). (5) The rate of dimerization has been shown to be much slower than the rate of autophosphorylation (*19*). (6) The enzyme has a substituted enzyme mechanism, with PEP and HPr as the two substrates (*3*, *4*, *20*, *21*).

Whether there is cooperativity between the two steps of the autophosphorylation of EI_2 by PEP has been an open question. Another unresolved issue is whether $([P]EI)_2$ dissociates to [*P*]EI before its phosphotransfer reaction with HPr (*13*, *22*). Such questions about mechanism are difficult to answer with steady state methods (*23*, *24*).

As the common component of all PTS systems, the first two reactions are fundamental to the physiology of many species of bacteria. A computer model has been developed that can predict the kinetic behavior of the glucose-specific

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[1] Abbreviations: PTS, bacterial phosphoenolpyruvate:glycose phosphotransferase system; Enzyme I, total protein, not specifying monomer or dimer; EI, monomer of Enzyme I; [*P*]EI, monomer phosphorylated at His189; EI_2, homodimer of Enzyme I; $([P]EI)_2$, doubly phosphorylated dimer; EI-N, amino-terminal domain of Enzyme I; EI-C, carboxy-terminal domain of Enzyme I; [*P*]HPr, HPr phosphorylated at His15; PEP, phosphoenolpyruvate; BSA, bovine serum albumin.

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PTS in *Escherichia coli* under a variety of conditions, *in vivo* and *in vitro* (*25*) (some of the data used to construct this model were preliminary estimates of rate constants reported here). Models such as this would aid in the understanding of many puzzling aspects of bacterial physiology, if data suitable to their construction were available. For instance, several important physiological phenomena are controlled by the state of phosphorylation of particular PTS proteins. One example is the activity of Mlc, a global repressor of those genes whose transcription is stimulated by glucose. The activity of this protein in *E. coli* is thought to be controlled by the state of phosphorylation of the glucose-specific PTS transporter, $IICB^{Glc}$ (*26*, *27*), thereby producing a response to the presence of glucose. However, the equilibria among all the proteins of the PTS through the common component HPr suggest that the presence of any PTS sugar should affect the state of phosphorylation of $IICB^{Glc}$. Similarly, the presence of non-PTS sugars was unexpectedly found to alter the state of phosphorylation of another protein of the glucose-specific PTS in *E. coli*, IIA^{Glc} (*28*), which is responsible for many of the regulatory functions of the PTS mentioned above. Quantitative data that would allow construction of a model of these observations do not exist, and answers to these questions will require accurate kinetic data for all the reactions of the PTS, especially their rate constants.

EXPERIMENTAL PROCEDURES

Materials. All buffer salts and other reagents were of purity typical for research reagents purchased from standard commercial sources. The pH of all buffers is reported at the temperature and concentration at which they were used.

Protein Assays. The method of Bradford (*29*) was used; the reagent was purchased from Bio-Rad Laboratories (Hercules, CA).

Purification of Proteins. Homogeneous Enzyme I was a gift from F. Chauvin and was purified by previously published methods (*17*). Homogeneous HPr was purified by previously published methods (*30*).

Sugar Phosphorylation Assay for PTS Activity. The PEP-driven sugar phosphorylation assay was performed as reported previously (*31*−*33*).

Assays for the Concentration of Enzyme I and HPr. The concentration of Enzyme I was measured as previously described (*17*). The concentration of HPr was measured by using the lactate dehydrogenase coupled assay (*31*, *32*) which measures the quantity of protein that can accept a phospho group from PEP. When HPr was phosphorylated in preparation for rapid quench assays, its concentration as estimated from the specific activity of the [^{32}P]PEP agreed with the results of the lactate dehydrogenase coupled assay within 5%.

Synthesis of [^{32}P]PEP. The enzymatic synthesis (*34*) was performed with modifications as described previously (*35*).

Preparation of [^{32}P]HPr. [^{32}P]HPr was prepared as described previously, with careful attention to the accuracy of the specific activity of the [^{32}P]PEP (*35*).

Rapid Quench Assays. This study employed the same rapid quench apparatus described previously (*35*), in addition to the same setup and use. The quench solution was 3 M KOH with 5 M urea, used as 1 volume of quench to 2 volumes of reaction mixture. Analysis of the quenched reactions by gel filtration chromatography using HPLC-grade columns at pH 12.3 was performed as described previously (*35*). The solution used to dilute the stock Enzyme I solution for the reactant syringe was the same as that in which the protein had been stored: 50 mM potassium phosphate buffer (pH 6.5), 1 mM EDTA, 0.2 mM DTT, 5 mM $MgCl_2$, and 0.5 mg/mL BSA. When an experiment used [^{32}P]PEP, the stock solution of [^{32}P]PEP was diluted with a solution of the same composition as that used for the Enzyme I; when HPr was added to such experiments, it was added as a 50−200-fold concentrated solution to the syringe containing the PEP. When an experiment used [^{32}P]HPr, it was diluted from the stock with 20 mM HCO_3^-/CO_3^{2-} (pH 9.5) and 1 mg/mL BSA, and the concentration of the phosphate buffer in the Enzyme I solution was increased to 100 mM. Preparation of the solutions for rapid quench experiments required large dilutions from stock solutions of Enzyme I and HPr, and a change from the frozen state to ambient temperature (from 23 to 26 °C), at which all experiments were performed. The solutions were therefore incubated for 1 h at ambient temperature before the experiment was started.

Enzyme I Monomer/Dimer Transition

$2EI \rightleftharpoons EI_2$:	k_A^*	k_D^*	K'_A

Phosphotransfer Reactions:

$PEP + EI_2 \rightleftharpoons Pyr + [P]EIEI$:	k_{Ia}	k_{-Ia}	I_a
$PEP + [P]EIEI \rightleftharpoons Pyr + ([P]EI)_2$:	k_{Ib}	k_{-Ib}	I_b
$([P]EI)_2 + HPr \rightleftharpoons [P]EIEI + [P]HPr$:	k_{II}	k_{-II}	II
$[P]EIEI + HPr \rightleftharpoons EI_2 + [P]HPr$:	k_{II}	k_{-II}	II
$EI + [P]HPr \rightleftharpoons [P]EI + HPr$:	k_{II}	k_{-II}	II

FIGURE 1: Monomer−dimer transition of Enzyme I described by forward and backward rate constants (k_A^* and k_D^*) and the association equilibrium constant, K'_A. The phosphotransfer reactions and their rate constants are numbered as in Rohwer et al. (*25*). The reported equilibrium constant for reaction I is 1.5 (*4*); k_{-I} was not measured in this study. The data impose some limits on the difference between k_{Ia} and k_{Ib} (see Results), but they are called k_I in the text. The measurements did not permit discrimination among rate constants of the three phosphotransfer reactions between Enzyme I and HPr (reactions II); the experimental data were fitted holding the rate constants equal.

Methods Used To Model the Experimental Data on the Rate of Phospho Group Transfer. The numerical integration program, Kinsim (*36*), as modified by Anderson et al. (*37*) was used to manually fit mathematical models to the experimental data. When experimental data met the criteria for nonlinear least-squares fitting of mathematical models, the Fitsim (*38*) module of Kinsim was used.

The model used for the kinetic analysis of the phosphotransfer reactions is shown as Scheme 1 in Figure 1. The convention used for numbering the reactions is adopted from Rohwer et al. (*25*). In this convention, the phosphotransfer reaction between HPr and IIA^{Glc} reported previously (*35*) would be numbered reaction III (not shown). The value of k_{-I} was fixed at 0.67 of the value of k_I, based on the equilibrium constant (1.5) for reaction I (*4*). The experimental

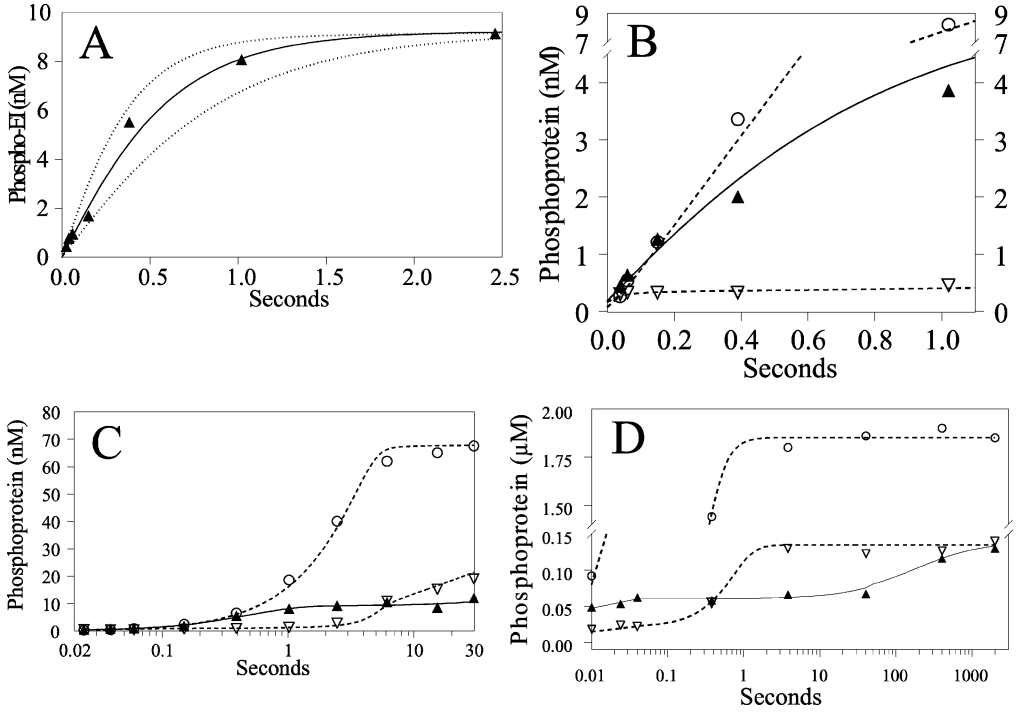

FIGURE 2: Transfer of the [^{32}P]phospho group from [^{32}P]PEP to Enzyme I alone and in the presence of HPr. Experimental data points and theoretical progress curves are shown for the transfer reactions. The rapid quench experiments were conducted as described in Experimental Procedures. The data points reflect the concentrations of phospho-EI; these concentrations are calculated as EI monomer to allow comparison with the initial total EI. The concentration of phosphorylated Enzyme I at the plateau in each experiment corresponds to the concentration of EI_2 present in the syringe before mixing as calculated from published values of K'_A, the equilibrium constant for the monomer−dimer transition. The same symbols are used in all the panels: (▲) [^{32}P]EI in the absence of HPr, (▽) [^{32}P]EI in the presence of HPr, (○) [^{32}P]HPr. (A) First 2.5 s of a progress curve shown on a linear time scale (no HPr). After mixing, the concentrations were as follows: 390 nM [^{32}P]PEP and 32.8 nM Enzyme I_{total} (24 nM EI and 4.4 nM EI_2). The rate constant, k_I, is shown in row 3 of Table 1. An estimate of the accuracy of the derived k_I is illustrated by the dotted lines; these curves were generated using k_I values of 1.5 or 0.67 times the value used to generate the curve shown as the solid line. (B) Two experiments are shown on a linear time scale, from 0 to 1 s. One consisted of 32.7 nM Enzyme I_{total} (27.3 nM EI and 2.7 nM EI_2), 370 nM [^{32}P]PEP, and no HPr. The second experiment was identical except that it also included 66 nM HPr. The rate constants that were obtained are given in rows 1 and 2 of Table 1. (C) Complete time course from the experiment in panel A on a logarithmic time scale. Time points longer than 4 s were obtained by hand mixing and timing. When present, HPr was at a concentration of 70 nM. The rate constants obtained by manual fitting are given in rows 3 and 4 of Table 1. (D) Two experiments are shown (with or without HPr) on a logarithmic time scale for 1800 s. Time points longer than 4 s were obtained by hand mixing and timing. After mixing, the concentrations were as follows: 44 μM [^{32}P]PEP, 140 nM Enzyme I_{total}, (79.4 nM EI and 30.3 nM EI_2) and 1.85 μM HPr (when present). The rate constants obtained by manual fitting are given in rows 6 and 7 of Table 1.

conditions made the model almost totally insensitive to the value chosen for k_{-1}.

RESULTS

Rates of Phosphotransfer from [^{32}P]PEP to EI_2 in the Absence of HPr. Previous work has shown that only EI_2 is active in reaction I (*3*−*9*); therefore, when both monomer and dimer are present, the progress curve for the autophosphorylation reaction is biphasic. Panels A and B of Figure 2 show the first few seconds of production of $([P]EI)_2$ at relatively low concentrations of [^{32}P]PEP and Enzyme I_{total}. Production reached a plateau in ~2 s under the conditions of these two experiments, whereas in an experiment that employed much higher concentrations of PEP and Enzyme I_{total}, the plateau was attained in less than 0.1 s (Figure 2D). The K'_A for the monomer−dimer transition required for optimization of the fit at the plateau concentration of $([P]EI)_2$ in the four experiments ranged from 1.8 to 4 × 10^6 M^{-1}; these values are in reasonable agreement with values of K'_A measured by analytical ultracentrifugation, 4.4−4.8 × 10^6 M^{-1} (*17*; H. Patel and K. Vyas, personal communication), under conditions similar to those used here.

The values for k_I from four experiments are summarized in Table 1 (rows 1, 3, 5, and 6). The concentrations of PEP and Enzyme I_{total} used in these experiments ranged from 0.37 to 44 μM and from 33 to 140 nM, respectively, showing that the modeled rate constants were independent of the concentrations of the reactants. As a measure of the error of fitting the data, Figure 2A shows the theoretical curves generated by Kinsim for values of k_I that are 1.5 times larger or smaller than the value chosen as the best fit. It is clear that the data allow manual fitting that is in error by a factor of less than 2. Similarly, the progress curve for reaction I in the absence of HPr shown in Figure 2B is well bracketed by 1.5-fold changes in k_I (not shown). A good fit of the experimental data from the early phase of the autophosphorylation of EI_2 by PEP was obtained by making k_{Ia} equal to k_{Ib}, but scatter in the data from some experiments makes it possible that k_{Ib} could range from $^1/_3$ to 3 times the value of k_{Ia} (not shown).

After the plateau had been reached, there was very little additional phosphorylation for the next 30 s (Figure 2C,D), but then phospho-Enzyme I continued to appear very slowly until all the enzyme present in the reaction mixture was phosphorylated, which took ~30 min under the conditions

Table 1: Rate Constants of the Phosphotransfer Reactions from [^{32}P]PEP to Enzyme I to HPr

Phosphotransfer between PEP and EI (with or without HPr)[a,b]						
row	[PEP] (nM)	$[EI]_{total}$ (nM)	[HPr] (nM)	k_I [$\times 10^{-6}$ M^{-1} s^{-1} ($\pm$SE)]	k_{II} [$\times 10^{-6}$ M^{-1} s^{-1} ($\pm$SE)]	k_{-II} [$\times 10^{-6}$ M^{-1} s^{-1} ($\pm$SE)]
1[c]	370	33	0	6		
2[c]	370	33	66	8	400	3.6
3[c]	388	33	0	8		
4[c]	388	33	68	10	200	2
5	753	66	0	6		
6[c]	44000	140	0	3.5		
7[c]	44000	140	1850	5	200	8
			mean	6.9 ± 2.2	266 ± 120	4.5 ± 3
Phosphotransfer between [^{32}P]HPr and EI[d]						
row	[EI] (nM)	[[*P*]HPr] (nM)	[HPr][e] (nM)		k_{II} [$\times 10^{-6}$ M^{-1} s^{-1} ($\pm$SE)]	k_{-II} [$\times 10^{-6}$ M^{-1} s^{-1} ($\pm$SE)]
8	50	50	7		210 ± 20[f]	3.7 ± 0.3
9[c]	50	31	19		190 ± 25	5.9 ± 0.3
10	50	31	15		120 ± 29	2 ± 0.4
				mean (rows 8−10)	173 ± 47	3.9 ± 2
				mean (rows 1−10)	220 ± 94	4.2 ± 2.4

[a] In our experiments, the K'_A required for fitting the curves ranged from 1.8 to 4×10^6 M^{-1}. There is no single K'_A for the EI−EI_2 transition. It can vary as much as 300-fold, depending on conditions such as temperature, ionic strength, pH, the presence or absence of its ligands, etc. (H. Patel and K. Vyas, personal communication). Under similar conditions, the value derived from the sedimentation equilibrium experiments was 4.8×10^6 M^{-1}. [b] Manual fitting of the data from four experiments. Rate constant k_{-I} was fixed at 0.67 times the value of k_I (see Experimental Procedures). [c] Experiments that are shown in the figures. [d] Fitting of the data from each experiment by the method of nonlinear least squares. [e] The HPr results from the hydrolysis of [*P*]HPr during storage. [f] The significance of the standard error of the individual rate constants that is produced by Fitsim is limited to whether it is less than one-fourth of the magnitude of the constant itself (*36*, *39*).

of the experiment shown in Figure 2D. This slow phase was modeled by rate constants for association of the Enzyme I monomer that are between two and three orders of magnitude smaller (not shown) than the rate constants of the fast phase of autophosphorylation. Thus, following the very rapid phosphorylation of the EI_2 present at mixing, the final stage of the time course represents the slow dimerization of the monomer and very rapid phosphorylation of the resulting dimer.

Rates of Phosphotransfer from PEP to Enzyme I in the Presence of HPr. The experiments described above that measured the rates of autophophorylation of Enzyme I were accompanied by a second set which included HPr in the reaction mixtures. The data from three experiments are summarized in Table 1 (rows 2, 4, and 7). The concentration of HPr in the three experiments ranged from 0.066 to 1.85 μM. In the presence of HPr, the rate of appearance of ([*P*]-$EI)_2$ is slower, and its concentration during the first few seconds of the reaction is much lower compared to the results in the absence of HPr (Figure 2B−D). The value of the rate constant, k_I, that produced the best fit was, however, unaffected by the presence of HPr. Although the concentrations of ([*P*]$EI)_2$ were very low, the theoretical fits to the experimental data points of both ([^{32}P]$EI)_2$ and [^{32}P]HPr were very sensitive to the absolute values of k_I and k_{II}. For example, either increasing or decreasing k_I from the value producing the best fit could not be compensated by a change in k_{II}. In other words, the values of k_I and k_{II} were tightly linked, and the values of k_I shown in Table 1 (rows 2, 4, and 7) represent independent measurements of the constant.

[^{32}P]HPr is produced at a very high rate during the initial period, with the values of rate constant k_{II} being $\approx 220 \times 10^6$ M^{-1} s^{-1} (see Discussion). Another important conclusion from the data shown in panels C and D of Figure 2 is that ([*P*]$EI)_2$ phosphorylates a significant molar excess of HPr in a short time. Figure 2C shows that 4.4 nM dimer phosphorylated 70 nM HPr, a 16-fold molar excess, in 6 s, while at higher concentrations in the experiment shown in Figure 2D, 30 nM dimer phosphorylated 1.85 μM HPr, a 60-fold molar excess, in ~1 s. These data strongly suggest that ([*P*]-$EI)_2$ does not undergo dissociation to the monomer before the transfer of its phospho group to HPr (see Discussion).

As the phosphorylation of HPr approaches completion, the concentration of ([*P*]$EI)_2$ begins to increase. Depending on the experimental conditions, this happens in a few tenths of a second (Figure 2D) to ~5 s (Figure 2C). The enzyme is phosphorylated much faster than it is in the absence of HPr, suggesting that EI (monomer) is phosphorylated in a back reaction by [*P*]HPr. In the experiment that utilized the highest concentration of reactants (Figure 2D), Enzyme I was phosphorylated ~1000-fold faster in the presence of HPr than in its absence. The values of the reverse rate constant, k_{-II}, determined from these three experiments ($\sim 4.5 \times 10^6$ M^{-1} s^{-1}) agree well with those obtained from the experiments presented in the next section ($\sim 3.9 \times 10^6$ M^{-1} s^{-1}) in which the phosphotransfer occurred between [*P*]HPr and both EI and EI_2.

Rates of Phosphotransfer between HPr and Enzyme I. Figure 3 shows a progress curve for the phosphotransfer reaction between [^{32}P]HPr and Enzyme I. Nonlinear least-squares analysis was used to fit the data from this and two other experiments; the rate constants that best fit the data are given in Table 1 (rows 8−10). In view of the unusually large value of k_{II}, we point out that in these experiments, k_{II} is determined by the late time points and k_{-II} is determined by the early ones, whereas in the experiments described in the previous section (in which the reactions were driven by PEP), the relationship is reversed, but the values are in good agreement.

The number of time points in the data sets was not sufficient for detection of small differences among the three rate constants of the reactions that are grouped as reaction II in Figure 1. It is clear from Figure 3, however, that a good

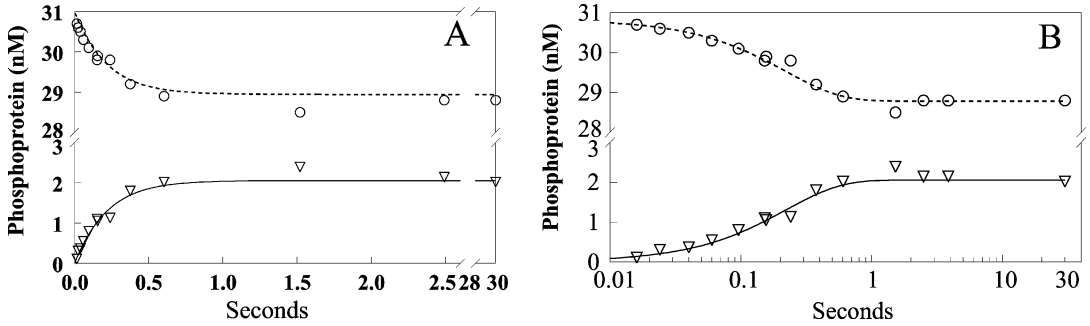

FIGURE 3: Transfer of the [^{32}P]phospho group from [^{32}P]HPr to Enzyme I. Experimental data points and theoretical progress curves are shown for the transfer reaction on a linear time scale (A) and a logarithmic time scale (B). The rapid quench experiments were conducted as described in Experimental Procedures. The data points reflect the concentrations of phospho-EI; these concentrations are calculated as EI monomer to allow comparison with the initial total EI. The time point after the break was obtained after hand mixing the solutions: (○) [^{32}P]HPr and (▽) [^{32}P]EI. At the instant of mixing, the concentrations were as follows: 31 nM [^{32}P]HPr, 18 nM HPr, and 50 nM Enzyme I. The rate constants obtained from nonlinear least-squares fitting are given in row 9 of Table 1.

fit to the data was obtained when the three constants were held equal to one another, and as shown in the preceding section, the phosphorylation of EI by [*P*]HPr is fit by a similar k_{-II}.

All of the rate constants reported in Table 1 differ slightly from the preliminary values used by Rohwer et al. (*25*) to construct a mathematical model of the glucose PTS. The differences are too small to impact the predictions of the model for control of the glucose PTS. The ratios of present value to preliminary value are as follows: $k_I = 6.9/6.0$, $k_{II} = 220/200$, and $k_{-II} = 4.2/8.0$. The model has low sensitivity to k_{-II}; the 2-fold change in this parameter will change the flux by approximately −4%.

DISCUSSION

We have used transient state kinetics to study the phosphotransfer reaction of the first two (general) steps of the PTS in *E. coli*: (1) the autophosphorylation of EI_2 by PEP and (2) the transfer of the phospho group from $([P]EI)_2$ to HPr. Our results strongly support many previous studies (*3*−*9*) that concluded that only EI_2 can be phosphorylated, and that the dimer accepts one phospho group per monomeric subunit. Our conclusions are based on the observation that the extent of the initial, rapid phosphorylation of the protein is predicted by the quantity of dimer calculated from previously measured equilibrium constants for the monomer−dimer transition (*17*, *18*; H. Patel and K. Vyas, personal communication).

The rapid phase of the phosphorylation of EI_2 is followed by a very slow rate of phosphorylation of the remaining enzyme. It has been shown that the rate of dimerization is orders of magnitude slower than the rate of phosphorylation (*19*, *40*). The progress curves of this phase from four experiments were successfully fit by rate constants of dimerization, k_A^*, that are between two and three orders of magnitude smaller than k_I, consistent with previously published results (*19*).[2] Therefore, the slow phase of phosphorylation corresponds to the slow dimerization of EI followed by very rapid phosphorylation of the dimer by PEP.

Addition of HPr to the phosphorylation reaction changes the patten of phosphorylation of Enzyme I. Phospho groups are transferred very rapidly from $([P]EI)_2$ to HPr (reaction II), and the high rate of this reaction lowers the concentration of $([P]EI)_2$ relative to that found in the absence of HPr. The forward rate constant of reaction II is more than 30 times larger that the forward rate constant of reaction I. The concentration of $([P]EI)_2$ remains low until almost all the HPr is phosphorylated; the remaining EI is then phosphorylated in a few seconds, and the slow phase of EI phosphorylation is absent. This can be explained by assuming that [*P*]HPr phosphorylates the EI monomer, an assumption consistent with the observation that the EI−N species (which cannot dimerize) is phosphorylated by [*P*]HPr (*12*, *16*). The data suggest that the rate constants, k_{-II}, for all of the three reactions included as reaction II in Figure 1 are equal, but the time points were not sufficiently dense to permit detection of small differences among them.

These second-order rate constants can be compared to kinetically identical constants derived from steady state kinetic data (*3*, *8*, *9*, *20*). The specificity constants, k_{cat}/K_m, of an enzyme with a ping-pong mechanism are identical to the second-order rate constants, k_I and k_{II} (*25*, *41*); i.e., for Enzyme I, $k_I = k_{cat}/K_{m(PEP)}$ and $k_{II} = k_{cat}/K_{m(HPr)}$. The previously reported steady state data require corrections for the estimated concentration of EI_2 (rather than Enzyme I_{total}) and for differences in the temperatures at which the reactions were measured. When these corrections are applied to the published values, k_I and k_{II} agree well with the respective specificity constants. Because the second-order rate constants, k_I and k_{II}, are affected by fewer systematic errors than k_{cat}/K_m, we think that k_I and k_{II} are the more accurate measures of reactions I and II, respectively.

An important question about the mechanism of Enzyme I is whether there is cooperativity between the successive autophosphorylation reactions of the monomeric subunits of the dimer; there are no existing data that bear on this question. The data presented here are sufficiently detailed to impose limits on the degree of cooperativity. Modeling of the data from two experiments suggests that the rate constant for the second step, k_{Ib}, is in the range of $0.33k_{Ia}$−$3k_{Ia}$. Seok et al. (*8*) and Garcia-Alles et al. (*42*) have shown that Enzyme I present largely as a monomer in a very dilute solution is activated by dimerization with a large excess of any of several Enzyme I mutants that are not in themselves active. The degree of activation approximates 100%, suggesting that an active monomer in a dimer with an inactive,

[2] None of the rate constants from Chauvin et al. (*19*) were obtained under conditions directly comparable to those used for the rapid quench experiments.

mutant monomer is fully active. This result is consistent with the suggestion that k_{Ia} and k_{Ib} are equal in the native dimer.

When, in the phosphotransfer reaction starting from PEP, HPr is present in excess over EI_2, the HPr is quantitatively phosphorylated at a rate that is far higher than that expected if the $([P]EI)_2$ first dissociated, transferred its phospho group to HPr, and then reassociated. In the experiment shown in Figure 2D, the EI_2 phosphorylated a 60-fold molar excess of HPr in ~1 s, whereas the EI in the mixture required 2000 s to become phosphorylated in the absence of HPr. The hypothesis of cyclic association and dissociation of Enzyme I (*22*) has recently been questioned on the basis of data from experiments using mutant Enzyme I (*13*).

In addition, the data presented here show that [*P*]HPr can phosphorylate the EI monomer; the phosphorylation of Enzyme I_{total} required ~3 s (Figure 2D) which is too fast for dimerization to have occurred. Weigel et al. (*4*) reported that Enzyme I was phosphorylated more rapidly in the presence of HPr. These two properties may have important implications for the physiology of the cell.

Dimitrova et al. (*18*) have suggested that PEP and Mg^{2+} induce a very high K'_A ($\sim 10^8$ M^{-1}) for the monomer−dimer transition, probably by reducing the rate constant for dissociation. The authors found that an unphosphorylatable active site mutant of Enzyme I (H189A) has a very high K'_A ($\geq 10^8$ M^{-1}) in the presence of Mg^{2+} and PEP; this K'_A is orders of magnitude larger than that of the wild type protein under the same conditions. It was suggested that the wild type protein responds similarly to the ligands, but the response is obscured by phosphorylation. Hübner et al. (*40*) have shown that Mg^{2+} and PEP decrease the rate of dissociation of EI_2 from *Staphylococcus carnosus*. The data in Figure 2D show that the rate of association of the wild type monomer is very slow, although both Mg^{2+} and PEP were present. Therefore, any increase in the K'_A of the wild type protein caused by these ligands must result from a decrease in k^*_D, in agreement with the conclusion of Hübner et al. (*40*).

The magnitude of k_{II}, the rate constant for the phosphotransfer from $([P]EI)_2$ to HPr, is unusually high for an interaction between two proteins that includes a covalent reaction. Two recent reviews of catalytically proficient enzymes (*43*, *44*) report such very large second-order rate constants for only binding reactions between proteins and macromolecular inhibitors (*45*, *46*), but none that involves a covalent reaction. If k_{II} were included in the list in Miller and Wolfenden (*44*), it would be the fourth largest second-order enzymatic rate constant involving covalent chemistry, ranking behind only those of superoxide dismutase with superoxide (7×10^9 M^{-1} s^{-1}), fumarase with fumarate (1×10^9 M^{-1} s^{-1}), and triose phosphate isomerase with glyceraldehyde 3-phosphate (4×10^8 M^{-1} s^{-1}), all of which are classified as diffusion-limited. The enzyme following in the list would be β-lactamase with penicillin (1×10^8 M^{-1} s^{-1}) which is classified as partially diffusion limited (*44*). These four enzymes catalyze reactions with small molecules.

The special significance of the reaction between phospho-Enzyme I and HPr, if it is diffusion-controlled, is that each contact between the two molecules is productive, involving phosphotransfer. How the active site, His15, of HPr (9.1 kDa) can be so accurately and instantaneously directed to the active sites of EI_2 (132 kDa) is not known. It suggests, however, that amino acid residues far from the active sites of both proteins must play an active role in the binding of the molecules, directing the proper orientation and rapid transfer of HPr along the surface of EI_2 to the active site, where phosphotransfer can take place.

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