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Escherichia coli Phosphoenolpyruvate-Dependent Phosphotransferase System: Equilibrium Kinetics and Mechanism of Enzyme I Phosphorylation†

Henk Hoving, Juke S. Lolkema, and George T. Robillard*

ABSTRACT: The phosphorylation of enzyme I from the *Escherichia coli* phosphoenolpyruvate-dependent phosphotransferase system was studied by means of isotope exchange between phosphoenolpyruvate and pyruvate. Experiments monitoring ^1H–^2H exchange showed that enzyme I phosphorylation is accompanied by the transfer of a proton from the enzyme to the C-3 atom of the substrate. ^{14}C–^{12}C-exchange experiments with both deuterated and protonated pyruvate exhibited a kinetic isotope effect (v_H/v_D = 1.9), showing that the proton transfer is (partly) rate determining and is an essential step in the mechanism of phosphoryl group transfer. Under certain reaction conditions, a more than proportional increase of the ^{14}C exchange rate with increasing total enzyme concentration was observed, indicating that only the dimeric form of enzyme I is phosphorylated. From the dependence of the ^{14}C exchange rate on the phosphoenolpyruvate and pyruvate concentrations, the forward and reverse second-order rate constants of the reaction were determined to be 3×10^7 and 8×10^5 M^{-1} min^{-1}, repsectively, yielding an equilibrium constant of ~40 and a $\Delta G°$ for enzyme I phosphorylation of −2.3 kcal/mol. The significance of the values of these rate constants for the thermodynamics of the phosphotransferase system is discussed.

Enzyme I, a component of the bacterial PEP[1]–dependent phosphotransferase system, catalyzes the transfer of a phosphoryl group from PEP to a phosphocarrier protein HPr. This is the first step in a process which ultimately leads to the phosphorylation and concomitant transport of PTS sugars into the bacterial cell (Roseman, 1969; Postma & Roseman, 1976; Hengstenberg, 1977). ^{32}P labeling experiments showed that enzyme I from *Staphylococcus aureus* reacts with PEP in the prescence of Mg^{2+} to give a phosphoenzyme capable of transferring its phosphoryl group to HPr (Stein et al., 1974). On the basis of these types of labeling experiments and kinetic data (Waygood & Steeves, 1980), the current view of the first two phosphotransferase reactions is

$$PEP + E_I \overset{Mg^{2+}}{\rightleftharpoons} E_IP + Pyr \quad (1)$$

$$E_IP + HPr \rightleftharpoons PHPr + E_I \quad (2)$$

This reaction scheme implies that isotope exchange[2] between PEP and pyruvate should be measurable in the presence of enzyme I and Mg^{2+}. In this paper we show that isotope exchange does occur at a rate comparable to the overall rate of sugar phosphorylation measured under the same conditions. This exchange process provides a useful assay of enzyme I in the absence of any other PTS components and concomitant impurities (enzyme II has not yet been purified). Because of the simplicity of the underlying chemical reaction, detailed studies of the kinetics of isotope exchange between PEP and pyruvate catalyzed by enzyme I may provide direct information about the thermodynamics and mechanism of enzyme I phosphorylation. These studies are now feasible since a procedure for the purification of enzyme I was developed recently in our laboratory (Robillard et al., 1979), pure enzyme being a prerequisite for unambigous results [Wimmer & Rose (1978) and references cited therein].

In the present paper we present kinetic studies of ^{14}C–^{12}C and ^1H–^2H exchange between PEP and pyruvate, catalyzed by enzyme I. The forward and reverse rate constants of reaction 1 were determined from the dependence of the ^{14}C-exchange rate on the PEP and pyruvate concentrations. $\Delta G°$ for enzyme I phosphorylation was calculated from the ratio of these two rate constants. ΔG values for reaction 1 were calculated as a function of the net reaction rate in steady state. The role of the monomeric and dimeric forms of enzyme I in the phosphorylation process was determined by measuring the enzyme concentration dependence of the isotope exchange rate. The mechanism of the conversion of the enol substrate (PEP) to the keto product (pyruvate) was studied by means of the exchange of the C-3 protons. ^{14}C-exchange experiments with both C-3 deuterated and protonated pyruvate demonstrated the rate-limiting nature of this proton transfer.

Materials and Methods

Chemicals. PEP (monopotassium salt and monocyclohexylammonium salt) and pyruvate (sodium salt) were purchased form Sigma Chemical Co. [1-^{14}C]PEP (monocyclohexylammonium salt; sp act., 5.0 mCi/mmol) was purchased from Amersham.

Enzyme I. Enzyme I was purified from *Escherichia coli* P650 by hydrophobic interaction chromatography (Robillard et al., 1979). The enzyme used in ^{14}C-exchange experiments was stored in 25 mM sodium phosphate, pH 7.2, at −20 °C. Enzyme I used in the NMR experiments was lyophilized and redissolved in D_2O 3 times in order to remove H_2O and was stored in lyophilized form at −20 °C. The enzyme I concentration was determined by measuring the amount of ^{32}P-labeled enzyme after reaction with ^{32}P-labeled PEP (O. Misset, unpublished data).

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[1] Abbreviations used: PEP, phosphoenolpyruvate; PTS, phosphotransferase system; DTT, dithiothreitol; NMR, nuclear magnetic resonance; FID, free induction decay; NADH, nicotinamide adenine dinucleotide hydride; LDH, lactate dehydrogenase; Pyr, pyruvate.

[2] The process under study is actually not the exchange of isotopes between PEP and pyruvate, but rather the interconversion of both. Nonetheless, the common term isotope exchange has been used throughout this paper, in accordance with the literature.

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Deuteration of Pyruvate. Pyruvate was deuterated by allowing a 100 mM solution of the sodium salt in D_2O containing 75 mM sodium phosphate and 1 mM sodium azide to stand at pH 7.4[3] and 4 °C for a few months. Before use in the isotope-exchange experiments, the pyruvate concentration was determined by measuring the NADH oxidation in an LD H assay. Even after 6 months the concentration was still over 95% of the original value. The deuteration at the C-3 atom takes place via the keto–enol tautomerization of pyruvate. At pH >11 the rate of keto–enol tautomerization is greatly enhanced and the C-3 protons are replaced by deuterons within a few hours at room temperature (90% deuteration at pH 11.2 in 4 h). Aldol condensation of the keto and enol forms at these high pH values can be prevented by using low pyruvate concentrations. In a 100 mM solution, 10–20% of the pyruvate appears as the aldol condensation product afater 4 h at pH 11.2, whereas no condensation product could be detected from a 10 mM solution under the same conditions. This rapid procedure was used for reprotonating deuterated pyruvate.

^{14}C-Exchange Experiments. All ^{14}C-exchange experiments were carried out in 25 mM sodium phosphate buffer, pH 7.1, containing 1 mM sodium azide and 0.5 mM DTT, at 37 °C. The complete reaction mixture, without the ^{14}C label, was preincubated for 5 min at 37 °C. The isotope-exchange process was started by adding an amount of ^{14}C-labeled PEP (1 mM solution in 10 mM sodium phosphate buffer, pH 7.1: sp act., 5.0 mCi/mmol) equal to 25% of the nonradioactive PEP (monocyclohexylammonium salt) already present during the preincubation. Samples between 100 and 200 μL were taken at several time intervals after the addition of the radioactive PEP, diluted 6–10-fold with cold water, and brought on 1 mL Dowex columns (Bio-Rad, AG 1-X2) in order to separate PEP from pyruvate. Pyruvate was eluted directly into scintillation vials with 5 mL of 125 mM lithium chloride solution, PEP was subsequently eluted into scintillation vials with 4 mL of 500 mM lithium chloride. The elution rate was ~0.5 mL/min, and the separation was always better than 95%. ^{14}C levels were measured in the presence of 8 mL of Packard emulsifier scintillator at 10 °C in a Nuclear Chicago Mark I scintiallation counter. The counting efficiency was 60%.

^{1}H-Exchange Experiments. As is shown above, the use of ^{14}C, a common lable in isotope-exchange studies, requires the separation of PEP from pyruvate for each data point. These manipulations can be circumvented by using proton NMR to monitor proton–deuteron exchange between PEP and pyruvate. In the proton NMR spectrum the C-3 proton resonances of PEP and pyruvate are well separated, and the change in signal intensities during the exchange can be directly followed, as is shown in figure 1. Exchange experiments were started with C-3 deuterated pyruvate and protonated PEP (monopotassium salt). The experiments were carried out in a 90 mM deuterophosphate buffer, pH 7.4, containing 1 mM sodium azide at 37 °C. The sample volume was 500 μL. Proton NMR spectra were recorded at 360 MHz on a Bruker HX 360 spectrometer, equipped with a Nicolet 1080 computer. The HOD resonance was supressed by gated decoupling at the HOD frequency. Thirty spectra were recorded consecutively in each kinetic experiment. Each spectrum was an average of 12–120 FID's, depending on the PEP, pyruvate, and enzyme I concentrations employed. The pulse repetition rate was 0.2 s^{-1}. All data were collected, stored on disc, processed, and integrated automatically by using the Nicolet NTCFT software.

[3] pD values are pH meter readings.

Theory

^{14}C–^{12}C- and ^{1}H–^{2}H-exchange experiments will be presented in the following sections. Only qualitative information will be drawn from the ^{1}H–^{2}H measurements because quantitative analysis is complicated by a H–D kinetic isotope effect (see below). However, a kinetic analysis of ^{14}C exchange is quite straightforward as will be shown in this section. Kinetic information concerning enzyme I (de)phosphorylation can be extracted from ^{14}C-exchange measurements if we express the kinetics of ^{14}C exchange in terms of the rate constants of the underlying chemical reaction.

A general reaction scheme must include the enzyme–substrate complexes:

$$\mathrm{PEP} + \mathrm{E_I} \underset{p_{-1}}{\overset{p_1}{\rightleftharpoons}} \mathrm{E_I{-}PEP} \underset{r_2}{\overset{p_2}{\rightleftharpoons}} \mathrm{E_IP{-}Pyr} \underset{r_1}{\overset{r_{-1}}{\rightleftharpoons}} \mathrm{E_IP} + \mathrm{Pyr} \quad (3)$$

Since steady-state approximations are valid for [E_I–PEP] and [E_IP–Pyr] (note that the system is not yet considered to be in chemical equilibrium), the overall chemical reaction obeys second-order kinetics:

$$\mathrm{PEP} + \mathrm{E_I} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{E_IP} + \mathrm{Pyr} \quad (4)$$

with

$$k_1 = \frac{p_1 p_2 r_{-1}}{(p_2 + p_{-1})(r_2 + r_{-1}) - r_2 p_2} \quad (5a)$$

and

$$k_{-1} = \frac{r_1 r_2 p_{-1}}{(p_2 + p_{-1})(r_2 + r_{-1}) - r_2 p_2} \quad (5b)$$

In a situation of chemical equilibrium, all concentrations are constant, including [E_I] and [E_IP]. Therefore, the transfer of the ^{14}C label obeys first-order kinetics in a chemical equilibrium:

$$^{14}\mathrm{C_{PEP}} \underset{k_{-1}^*}{\overset{k_1^*}{\rightleftharpoons}} {}^{14}\mathrm{C_{Pyr}} \quad (6)$$

with

$$k_1^* = k_1[\mathrm{E_I}] \qquad k_{-1}^* = k_{-1}[\mathrm{E_IP}] \quad (7)$$

Thus, the first-order rate constants are proportional to the concentrations of uncomplexed E_I and E_IP. The rate equations for eq 6 can be solved easily, yielding, with the boundary condition [$^{14}C_{Pyr}$] = 0 at t = 0

$$[^{14}\mathrm{C_{PEP}}] = \frac{[^{14}\mathrm{C_{PEP}}]^{t=0}}{[\mathrm{Pyr}] + [\mathrm{PEP}]}\{[\mathrm{Pyr}] \exp[-(k_1^* + k_{-1}^*)t] + [\mathrm{PEP}]\} \quad (8a)$$

$$[^{14}\mathrm{C_{Pyr}}] = \frac{[^{14}\mathrm{C_{PEP}}]^{t=0}}{[\mathrm{Pyr}] + [\mathrm{PEP}]}\{-[\mathrm{Pyr}] \exp[-(k_1^* + k_{-1}^*)t] + [\mathrm{Pyr}]\} \quad (8b)$$

From equations 8a and 8b it is clear that a logarithmic plot of the final value minus the measured values of either [$^{14}C_{PEP}$] or [$^{14}C_{Pyr}$] as a function of time directly yields $k_1^* + k_{-1}^*$ (see also Figure 3). The ratio k_1^*/k_{-1}^* can be determined from the chemical equilibrium equation

$$\frac{k_1^*}{k_{-1}^*} = \frac{k_1[\mathrm{E_I}]}{k_{-1}[\mathrm{E_IP}]} = \frac{[\mathrm{Pyr}]}{[\mathrm{PEP}]} \quad (9)$$

and, from the sum and the ratio, both first-order rate constants can be determined. This approach differs from the common

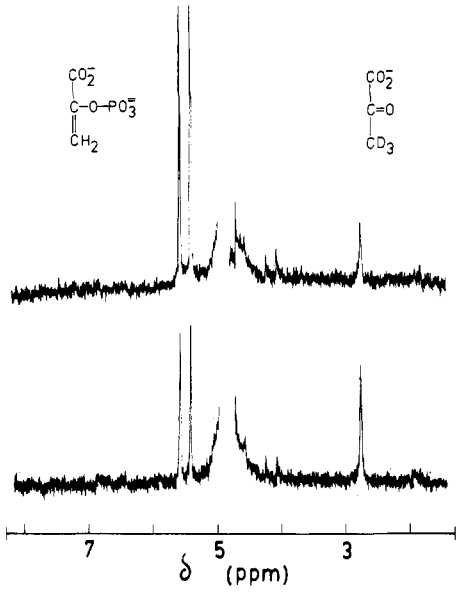


FIGURE 1: 360-MHz proton NMR spectra of a solution containing PEP, D-pyruvate, enzyme I, and $MgCl_2$; (Upper spectrum) Shortly after the start of an exchange experiment; (lower spectrum) after equilibrium of the degrees of protonation has been achieved.

technique of measuring initial velocities of isotope exchange in that it is based on a fit of the whole course of the exchange process, instead of the slope near $t = 0$. The advantage of this method is that it reduces the error in the determined values of the rate constants (compare Figure 3). This method was used in all ^{14}C-exchange experiments. Thus, each experimental k_1^* or k_{-1}^* value in the following sections originates from a time course as represented in Figure 3 and is derived from it as described in this section.

Results

Proton Transfer. Phosphorylation of enzyme I from PEP yields keto pyruvate as a reaction product at neutral pH. Information concerning the mechanism by which the enol substrate (PEP) is converted to the keto product (pyruvate) can be obtained from isotope-exchange experiments with the C-3 protons as exchange labels. These experiments were carried out by starting with C-3 deuterated pyruvate and protonated PEP and then measuring the proton NMR intensities of both as a function of time. As the exchange process proceeds, protonated PEP is converted to protonated pyruvate causing an increase in the pyruvate proton resonance intensity, and, concomitantly, deuterated pyruvate is converted to deuterated PEP causing a decrease in the intensity of the PEP proton resonance intensity (see Figure 1). When a large number of spectra are collected over short time intervals during the exchange process and the NMR intensities are plotted as a function of time, we obtain an accurate picture of the kinetics of the exchange process as shown in the lower portion of Figure 2.

It is important to note that the ratio $[E_I]/[E_IP]$ reaches its equilibrium value virtually immediately upon addition of PEP and pyruvate. The concentrations of PEP and pyruvate will not noticeably change upon establishment of chemical equilibrium because they are ~1000-fold higher than the total enzyme concentration. Therefore the time courses depicted in Figure 2 represent the interconversion of PEP and pyruvate at chemical equilibrium.

The proton NMR intensity of pyruvate depicted in Figure 2 arises from the keto form; the enol form is not observed because the equilibrium between both tautomers is far to the keto side at neutral pH. A careful analysis of the data in

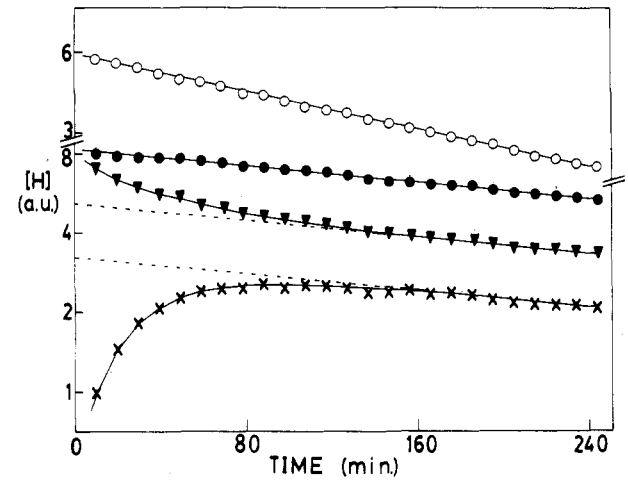


FIGURE 2: (Lower portion) H–D exchange between PEP (▼) and pyruvate (×) and the decrease of the total C-3 proton concentration (●); the reaction mixture contained 30 mM PEP, 15 mM pyruvate (deuterated at $t = 0$), 3 μM enzyme I, and 10 mM $MgCl_2$. (Upper portion) Deuteration of 15 mM pyruvate (protonated at $t = 0$) under the same conditions but in the absence of PEP.

Figure 2 will reveal whether the keto or the enol form of pyruvate is the immediate product of enzyme I phosphorylation:

$$\text{PEP} + E_I \overset{Mg^{2+}}{\rightleftharpoons} E_IP + \text{keto-Pyr}$$

$$\text{keto-Pyr} \overset{D_2O}{\rightleftharpoons} \text{enol-Pyr} \qquad (10a)$$

$$\text{PEP} + E_I \overset{Mg^{2+}}{\rightleftharpoons} E_IP + \text{enol-Pyr}$$

$$\text{enol-Pyr} \overset{D_2O}{\rightleftharpoons} \text{keto-Pyr} \qquad (10b)$$

Since an enol substrate is in equilibrium with a keto product (the observed interconversion is between PEP and keto pyruvate), a proton or deuteron must enter into the reaction, either in the first step (reaction 10a) or in the second (reaction 10b). In reaction 10b, a solvent deuteron participates in the interconversion of PEP and keto pyruvate, since every molecule of enol pyruvate released from E_IP in the forward reaction will pick up a solvent deuteron in the conversion to the keto tautomer. This is certainly not consistent with the observed difference between the rate of interconversion of PEP and keto pyruvate on the one hand (see the first 60 min in Figure 2) and the deuteration rate on the other hand (see the final decrease of the degrees of protonation or the decrease of the total proton concentration of PEP and pyruvate (●) in Figure 2). In other words, a molecule of keto pyruvate is converted to PEP and back to keto pyruvate more often than a proton or deuteron is lost to the solvent. It can therefore be concluded that the keto form of pyruvate is the immediate product of enzyme I phosphorylation.

Since, in the forward reaction, the extra C-3 proton (or deuteron) is not taken up from the solvent (see discussion above), we must conclude that it is abstracted from the enzyme and that this proton on the enzyme does not rapidly exchange with the solvent. This point is also illustrated by the fact that the gradual decrease of the degrees of protonation of pyruvate and PEP, when they are in equilibrium, is so much slower than the deuteration of pyruvate in the absence of PEP (see upper portion of Figure 2). In both cases this deuteration is caused by the keto–enol tautomerization of pyruvate in D_2O, and the data are only consistent if it is assumed that the first step in reaction 10a does not cause any noticeable loss of protons to

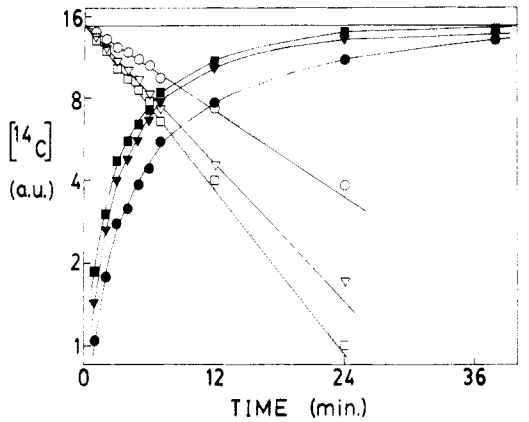


FIGURE 3: Rate of ^{14}C labeling of pyruvate from PEP, with C-3 protonated pyruvate (■), C-3 deuterated pyruvate (●), and reprotonated pyruvate (▼); the open symbols represent the differences between the final and the measured values; the samples contained 50 μM PEP, 750 μM pyruvate, 53 nM enzyme I, and 2.5 mM $MgCl_2$.

the solvent (i.e., if the reactive protons on the enzyme do not exchange with solvent deuterons at a significant rate). With this assumption the proton transfer is properly described by the reaction equation:

$$H_{PEP} \overset{\text{fast}}{\rightleftharpoons} H_{\text{keto-Pyr}} \overset{\text{slow}}{\underset{k}{\rightarrow}} \text{solvent} \qquad (11)$$

The rate equation for the loss of protons to the solvent now becomes

$$\frac{d}{dt}([H_{PEP}] + [H_{Pyr}]) = -k[H_{Pyr}] \qquad (12)$$

Since the first step in reaction 11 is much faster than the second one, the degrees of protonation of PEP and pyruvate will, to a good approximation, become equal

$$\frac{[H_{PEP}]}{[H_{Pyr}]} = \frac{2}{3}\frac{[PEP]}{[Pyr]} \qquad (13)$$

This is consistent with the data in Figure 2. Substituting eq 13 into eq 12, one finds that $[H_{PEP}]$ and $[H_{Pyr}]$ decrease with the same first-order rate constant λ (compare $[H_{PEP}]$ and $[H_{Pyr}]$ in Figure 2 after 160 min) with

$$\lambda = \frac{k}{1 + \frac{2}{3}\frac{[PEP]}{[Pyr]}} \qquad (14)$$

In eq 12 and 14 k represents the first-order rate constant for the deuteration of pyruvate (compare eq 11), which can be measured in the absence of PEP. From the upper line in Figure 2, k is determined to be 0.0040 min^{-1}. Substitution of this value into eq 14 yields, with [PEP]/[Pyr] = 2, λ = 0.0019 min^{-1}, which is in excellent agreement with the value determined experimentally, λ = 0.0018 min^{-1} (see lower portion of Figure 2). This result confirms the conclusion that the C-3 protons are preserved during the interconversion of PEP and keto pyruvate via enzyme I.

Kinetic Isotope Effect. In the preceding section we showed that phosphorylation of enzyme I includes the transfer of a proton from the enzyme to PEP. This finding prompted us to determine if this proton-transfer step was rate limiting. Since a kinetic H–D isotope effect would prove this step to be rate limiting, ^{14}C–^{12}C-exchange experiments were performed with both C-3 deuterated and protonated pyruvate. Figure 3 shows the increase of [^{14}C]pyruvate in experiments started with labeled PEP and unlabeled protonated (■) and deuterated (●) pyruvate. A logarithmic plot of the differences between the final and the measured label concentrations as a function of time yields a straight line, the slope of which is equal to the sum of the forward and reverse first-order rate constants of the ^{14}C-exchange process (see Theory). The data clearly show a kinetic isotope effect, v_H/v_D = 1.9. Note that in the experiment with deuterated pyruvate the enzyme is immediately deuterated at t = 0, because the total enzyme concentration is a few orders of magnitude smaller than the concentration of pyruvate. Also, the catalytic residue on the enzyme and the C-3 atom of pyruvate remain deuterated during the course of the ^{14}C-exchange measurement, because the concentration of deuterated pyruvate added to the reaction mixture is 15 times the concentration of protonated PEP.

As an extra control, the 100 mM deuterated pyruvate solution used was diluted 10-fold in H_2O and reprotonated at pH 11.7 as described under Materials and Methods and was used in another ^{14}C-exchange experiment (▼). As can be seen in Figure 3, the exchange rate returns to nearly the original value when this 80–90% reprotonated pyruvate is used.

Enzyme I Dimer. Molecular weight studies of enzyme I revealed that the enzyme reversibly dissociates from a dimeric to a monomeric form, with molecular weights of approximately 140 000 and 70 000, respectively. (Misset et al., 1980; Waygood & Steeves, 1980). Kinetic studies monitoring HPr phosphorylation (Waygood et al., 1979) and sugar phosphorylation (Misset et al., 1980) indicated that only the dimeric form of enzyme I is able to transfer a phosphoryl group from PEP to HPr. With the isotope-exchange method described in this paper, it is possible to investigate the influence of the state of aggregation of the enzyme on the phosphorylation only (i.e., reaction 1 instead of reactions 1 plus 2). In general, if the enzyme I concentration is low enough, the enzyme will exist almost completely in the monomeric form and, as a consequence, the dimer concentration will increase quadratically with the total enzyme concentration. If, in this case, only the dimer can be phosphorylated, the isotope-exchange rate will also increase quadratically with the total enzyme concentration. If, however, the monomer can be phosphorylated at the same rate, the isotope-exchange velocity will remain linearly dependent on the total enzyme concentration.

Lower enzyme I concentrations can be achieved in the ^{14}C-exchange experiments than in the ^{1}H-exchange experiments, because the NMR detection method requires millimolar concentrations of PEP and pyruvate for adequate signal to noise ratios and hence a rather high enzyme I concentration (micromolar) for an appropriate exchange rate (compare Figures 2 and 3). Therefore, the ^{14}C method was applied to the enzyme concentration dependent studies. As shown in Figure 4A, the exchange rate increases more than proportionally with the enzyme concentration, especially at low Mg^{2+} concentrations. Figure 4B shows that this is consistent with a model in which only the enzyme I dimer can be phosphorylated, as in the sequence

$$\begin{array}{l} \text{PEP} + E_I \underset{p_{-1}}{\overset{p_1}{\rightleftharpoons}} E_I\text{–PEP} \underset{r_2}{\overset{p_2}{\rightleftharpoons}} E_IP\text{–Pyr} \underset{r_1}{\overset{r_{-1}}{\rightleftharpoons}} E_IP + \text{Pyr} \\ \quad {\scriptstyle K_D}\big\| \\ \quad 2E_I{}^{\text{mon}} \end{array} \qquad (15)$$

In this model

$$[E_I]^{\text{tot}} = [E_I] + [E_IP] + [E_I\text{–PEP}] + [E_IP\text{–Pyr}] + \tfrac{1}{2}[E_I{}^{\text{mon}}] \qquad (16)$$

All concentrations on the right hand side of eq 16 can be

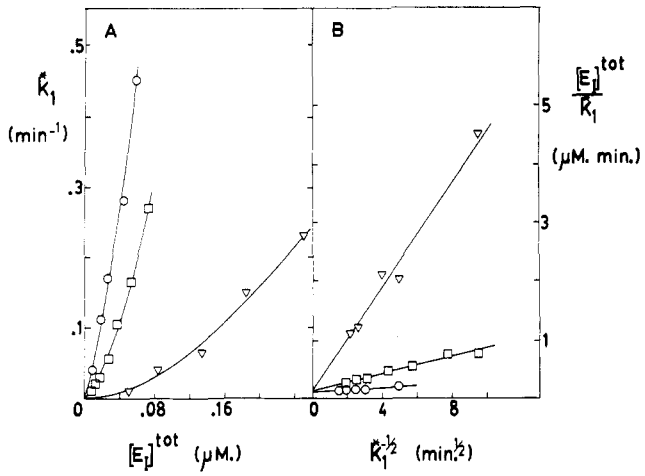


FIGURE 4: (A) Rate of ^{14}C exchange between PEP (50 μM) and pyruvate (1250 μM) as a function of the total enzyme I concentration, at different Mg^{2+} concentrations: 0.25 mM (▽); 2.5 mM (□); 25 mM (○). (B) Data of (a) replotted.

expressed in $[E_I]$ from the chemical equilibrium equations, yielding

$$[E_I]^{tot} = k_1[E_I]\left\{\frac{1}{k_1} + \frac{1}{k_{-1}}\frac{[PEP]}{[Pyr]} + [PEP] \times \left(\frac{p_1}{k_1 p_{-1}} + \frac{r_1}{k_{-1}r_{-1}}\right) + \tfrac{1}{2}\left(\frac{K_D}{k_1}\right)^{1/2}(k_1[E_I])^{-1/2}\right\} \quad (17)$$

From eq 17 and 12 it can be seen that a plot of $[E_I]^{tot}/k_1^*$ vs. $k_1^{*-1/2}$ at fixed PEP and pyruvate concentrations should yield a straight line with a positive intercept, as was found expermentally (Figure 4B).

The deviation from linearity decreases with increasing Mg^{2+} concentration, showing that in some way Mg^{2+} shifts the monomer–dimer equilibrium to the dimer side. The exact mechanism of the interaction with Mg^{2+} is not yet known, but studies are in progress. Since the effect of Mg^{2+} is not included in reaction 15, the K_D defined there is only an apparent dissociation constant which is a function of the Mg^{2+} concentration (see Figure 4B). The effect of temperature and pH on the apparent dissociation constant has been reported in earlier studies (Waygood et al., 1979).

Second-Order Rate Constants. The values of the rate constants k_1 and k_{-1} can be determined from the influence of the ratio [PEP]/[Pyr] on k_1^* or k_{-1}^*, if all of the enzyme exists in the uncomplexed E_I and E_IP forms. As can be seen from eq 17, a plot of $1/k_1^*$ vs. [PEP]/[Pyr] directly yield the values of k_1 and k_{-1}, if the last three terms in the equation (representing the last three terms in eq 16) are negligible. The amount of monomeric enzyme I is negligible at 25 mM Mg^{2+} (see Figure 4), and the amounts of E_I–PEP and E_IP–Pyr will become negligibly small at sufficiently low PEP and pyruvate concentrations. These conditions cannot be achieved in the ^1H-exchange experiments because of the low sensitivity of the NMR detection method, and, therefore, the exchange studies for the determination of the second-order rate constants were performed with ^{14}C as a label.

The dependence of k_1^* on the ratio [PEP]/[Pyr] at infinitely low PEP and pyruvate concentrations can be determined by varying the PEP and pyruvate concentrations at each fixed ratio [PEP]/[Pyr] and extrapolating back to zero concentrations. According to eq 17, plots of $1/k_1^*$ vs. [PEP] at different [PEP]/[Pyr] ratios should yield a series of parallel straight lines. Figure 5A shows that this is not the case but that the slope increases with decreasing [PEP]/[Pyr]. A possible explanation for this is competitive product inhibition. If pyruvate binds to E_I, a term $[E_I$–Pyr] should be added to the right hand side of eq 16, yielding a term $[E_I][Pyr]/K_D^{E_I\text{-}Pyr}$ in eq 17 and hence a term $(1/E_I^{tot})(1/k_1K_D^{E_I\text{-}Pyr})([Pyr]/[PEP])$ in the slope of the aforesaid plots. We wish to note here that ^1H-exchange experiments at [PEP]/[Pyr] = 0.5–4 showed an increase of this slope with increasing [PEP]/[Pyr], indicating that competitive binding of PEP to E_IP occurs as well.

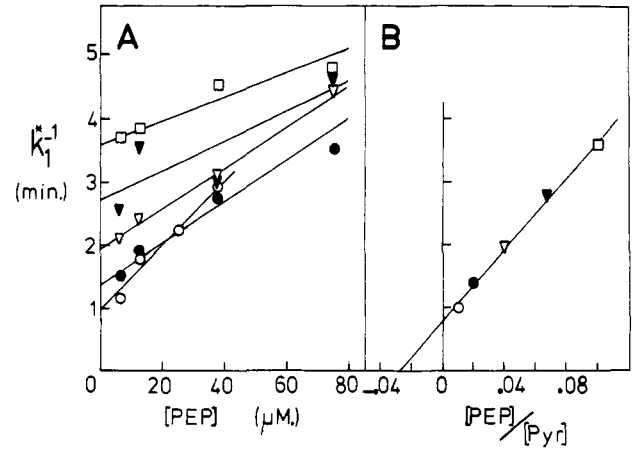


FIGURE 5: (A) Reciprocal plot of the forward rate constant of ^{14}C exchange between PEP and pyruvate as a function of [PEP] at different [PEP]/[Pyr] ratios: 0.01 (○), 0.02 (●), 0.04 (▽), 0.07 (▼) and 0.10 (□); the experiments were carried out with 44 nM enzyme I in the presence of 25 mM $MgCl_2$. (B) Intercepts of (A) plotted against the [PEP]/[Pyr] ratio.

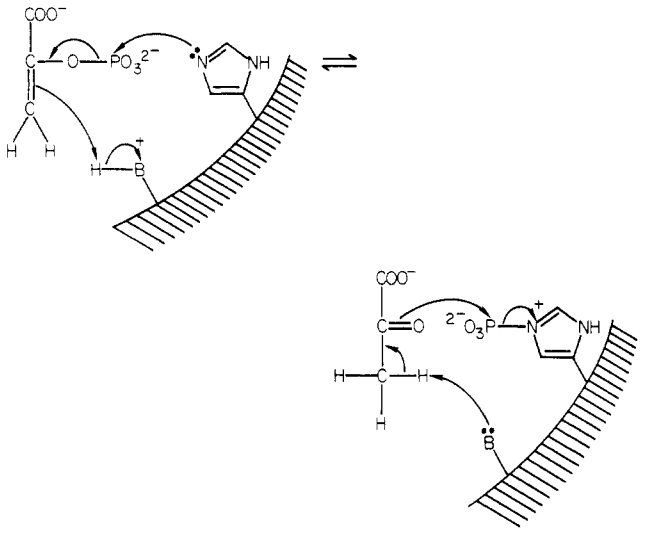
The intercepts in Figure 5A are plotted against the ratio [PEP]/[Pyr] in Figure 5B, yielding a straight line with slope $1/(k_{-1}[E_I]^{tot})$ and intercept $1/(k_1[E_I]^{tot})$ (see eq 17). The values for k_1 and k_{-1} thus obtained are 3×10^7 M^{-1} min^{-1} and 8×10^5 M^{-1} min^{-1}, respectively. It is important to note that these values correspond with the enzyme I concentration expressed as a concentration of active sites, determined by ^{32}P labeling (see Materials and Methods). From this, the equilibrium constant of reaction 1, which is independent of the definition of the enzyme concentration, is ~40, and the corresponding $\Delta G°$ is −2.3 kcal/mol.

Discussion

Kinetic Relevance of Isotope Exchange. The isotope-exchange process studied is based upon the phosphorylation (and dephosphorylation) of enzyme I. Neither the isolation of the phosphorylated enzyme (Stein et al., 1974) nor the observation of isotope exchange in itself proves that the PTS reaction proceeds via a phosphoenzyme I intermediate [see Wimmer & Rose (1978) and references cited therein]. Isotope exchange between PEP and pyruvate should occur at a rate comparable to the rate of the overall PTS reaction for the formation of phosphoenzyme I to be a possible step in the PTS reaction pathway. It is possible from kinetic studies of sugar phosphorylation to make a reliable estimate of the amount of free E_I present in the steady state. This value, combined with the PEP concentration in the assay and the steady-state rate of sugar phosphorylation, yields a value on the order of 10^7 M^{-1} min^{-1} for the second-order rate constant of the reaction of PEP with free E_I in the complete PTS assay (O. Misset, personal communication). Comparison of this value with that of k_1 from the isotope-exchange studies shows that the exchange process is indeed kinetically relevant.

Proton Transfer. The proton-exchange data presented in this paper clearly show that the transfer of a proton from enzyme I to the C-3 atom of PEP is involved in enzyme I phosphorylation. Since pyruvate appears to dissociate from

Scheme I: (De)phosphorylation of Enzyme I



phosphoenzyme I only in the keto form, it is possible that phosphoryl group and proton transfers are one concerted process, as indicated in Scheme I.[4] The kinetic H–D isotope effect shows that the proton transfer is (partly) rate determining in this reaction. The chemical nature of B in Scheme I has not yet been determined. A histidine (possibly the one which becomes phosphorylated) or a sulfhydryl group is a possible candidate. Whatever its nature, however, it is remarkable that its proton does not rapidly exchange with solvent during the course of a reaction. More information about the amino acid composition and the structure of the active site will be required for a satisfactory explanation of this phenomenon. Another interesting question raised by the observation of the proton transfer is how the catalytic residue becomes reprotonated when E_IP reacts with HPr in the normal PTS reaction sequence.

Enzyme I Dimer. The more than proportional increase of the ^{14}C-exchange rate with the total enzyme concentration is consistent with the model proposed by Misset, in which only dimer enzyme I can be phosphorylated (Misset et. al., 1980). The determination of the dissociation constant has to await further studies of the interaction with divalent metal ions, which are presently in progress. Although the dissociation constant is not yet known, the data now available do suggest that enzyme I exists solely in the dimeric form under physiological conditions. The total enzyme I concentration in the cell is estimated to be in the micromolar range. If we assume that the monomer–dimer equilibrium is not influenced by other cellular components, then we can conclude from Figure 4 that the enzyme will be almost completely dimerized, even at low Mg^{2+} concentrations.

Relation between Rate Constants and Thermodynamics of the PTS. The $\Delta G°$ of enzyme I phosphorylation from PEP was found to be −2.3 kcal/mol. The $\Delta G°'$ (standard H^+ concentration, 10^{-7}M) of PEP hydrolysis is −14.8 kcal/mol, yielding a value of −12.5 kcal/mol for $\Delta G°'$ of phosphoenzyme I hydrolysis.

It is important to notice that the $\Delta G°$ value of a chemical reaction does not contain any information concerning the efficiency of the reaction in terms of Gibbs free energy preservation. $\Delta G°$ only defines the equilibrium position, and the actual loss of Gibbs free energy ($-\Delta G$) depends on how far the system is away from equilibrium (i.e., how irreversibly in the thermodynamic sense the reaction proceeds). It will be clear that the higher the values of the forward and reverse rate constants, the smaller the deviation from equilibrium required for a certain net reaction rate in either direction. The dependence of ΔG on the net reaction velocity is easily derived for a simple reaction such as eq 9 [see, also, Crabtree & Taylor (1979)]

$$\Delta G = -RT \ln \frac{k_1}{k_{-1}} + RT \ln \frac{[E_IP][Pyr]}{[E_I][PEP]} = RT \ln \frac{v^-}{v^+} \quad (18)$$

With a net forward reaction, the velocity $v = v^+ - v^-$ and ΔG becomes

$$\Delta G = RT \ln \left(1 - \frac{v}{v^+}\right) \quad (19)$$

It is this ΔG value which has to be calculated for an appropriate discussion of the efficiency of the corresponding step in the PTS.

The PTS being a sugar transport system, the ΔG values of the component reactions at a certain net reaction rate directly influence the sugar gradient which can be maintained across the cell membrane at that particular rate of sugar uptake. The ΔG values of the component reactions add up to the total ΔG of the overall PTS reaction which is

$$\Delta G_{PTS} = \Delta G°_{PTS} + RT \ln \frac{[Pyr]}{[PEP]} \frac{[Glc\text{-}6\text{-}P_{in}]}{[Glc_{out}]} = RT \ln \frac{grad}{grad^{eq}} \quad (20)$$

In this equation the glucose gradient (grad) is defined as grad = $[Glc\text{-}6\text{-}P_{in}]/[Glc_{out}]$; $grad^{eq}$ is this value at equilibrium and can be calculated from $\Delta G°_{PTS}$ (−11.5 kcal/mol) and [PEP]/[Pyr]:

$$grad^{eq} = \frac{[PEP]}{[Pyr]} \exp(-\Delta G°_{PTS}/RT) \quad (21)$$

The influence of the ΔG values of the component PTS reactions at a certain PTS reaction rate on the sugar gradient across the cell membrane can now be described as follows:

$$\Delta G_{PTS}(v) = \sum_i \Delta G_i(v) \quad (22a)$$

or

$$\exp[\Delta G_{PTS}(v)/RT] = \prod_i \exp[\Delta G_i(v)/RT] \quad (22b)$$

Substitution of eq 19 and 20 into eq 22 yields

$$\frac{grad(v)}{grad^{eq}} = \prod_i (1 - v/v_i^+) \quad (23)$$

After a thorough study of the kinetics of enzyme I phosphorylation, we would like to be able to calculate ΔG for this step in the PTS reaction (and hence the corresponidng term in eq 23) under physiological conditions and at rates of sugar uptake by the bacterial cell under various growth conditions. Such calculations are, however, hampered by the lack of detailed kinetic information concerning the second reaction in which enzyme I is involved; the reaction of phosphoenzyme I with HPr. Calculation of v^+ for the first reaction requires knowledge of the amount of free E_I, which also depends on the amount of the E_IP–HPr complex present. The amount of E_IP–HPr can be estimated by assuming that the K_D for this complex equals the K_M of 10 μM (Waygood & Steeves, 1980)

[4] Phosphorylation of enzyme I is assumed to take place at the N-3 of a histidine residue, according to the results of Hengstenberg on *S. aureus* enzyme I (Hengstenberg, 1977).

Table I: ΔG of Enzyme I Phosphorylation at Different PTS Reaction Rates

v (μM/min)	$[E_I]$ (μM)	$[E_IP]$[a] (μM)	$1 - v/v^+$	ΔG (kcal/mol)
0	0.15	1.50	1	0
40	0.16	1.48	0.92	−0.05
800	0.39	1.26	0.32	−0.68

[a] It was assumed that the sum of the $E_I + E_IP$ concentrations does not change significantly.

and that the value of the equilibrium constant for the phosphorylation of HPr from E_IP is on the order of unity (Hengstenberg, 1977).

A few tentative calculations are presented in Table I. The total enzyme I and HPr concentrations in the cell are estimated to be 2 and 25 μM, respectively. Reasonable estimates for the PEP and pyruvate concentrations in the cell are 100 and 400 μM, respectively (Lowry et al., 1971). Figure 5 shows that under these conditions the amounts of enzyme I in the complex forms with PEP and pyruvate are negligible. From the data and assumptions presented above, the amounts of E_I and E_IP in a chemical equilibrium situation are calculated to be 0.15 and 1.5 μM, respectively. Hence the forward and reverse rates of enzyme I (de)phosphorylation at equilibrium are $v^- = v^+ = k_1[PEP][E_I] = 450$ μM/min. Starting from this value, ΔG of enzyme I phosphorylation can be calculated for a certain net reaction rate equal to the steady-rate of sugar uptake via the PTS. Estimates for the rate of sugar uptake vary between 40 and 800 μM/min (D. Tempest, personal communication), and the corresponding ΔG values are given in Table I. These ΔG values represent the actual loss of Gibbs free energy, caused by the thermodynamic irreversibility of enzyme I phosphorylation at the corresponding net reaction rates. The decrease of the maximum attainable sugar gradient, caused by this loss of Gibbs free energy, is represented by the $(1 - v/v^+)$ term. The maximum gradient at $[PEP]/Pyr] = {}^1/_4$ equals 28×10^6 (see eq 21). Thus at a PTS reaction rate of 800 μM/min, the "friction" in the enzyme I phosphorylation step causes a loss of Gibbs free energy of 0.7 kcal/mol[5] and a concomitant decreases of the maximum attainable sugar gradient to 9×10^6. This value will not be attained, because the other stepts in the PTS will have similar effects. These effects can be calculated as soon as sufficient kinetic data are available. An isotope-exchange study concerning the E_{II}-catalyzed reaction of the *Bacillus subtilis* PTS was published recently (Perret & Gay, 1979).

In conclusion, we can say that sufficient kinetic data will enable us to give a complete analysis of the efficiency of the PTS and of its component reactions. In this discussion section we presented an example of such an analysis concerning the enzyme I phosphorylation step, on the basis of kinetic data from isotope-exchange studies.

[5] Although the ΔG values calculated here do not necessarily represent the dissipation of energy (Crabtree & Taylor, 1979), they are in some way related to this. This can be seen by considering the reaction as a part of some cyclic biological process. In such a cyclic process the sum of the ΔG's of the component reactions has to be matched by the input of an equal amount of "work" (radiation energy from the sun), which is dissipated in the overall process.

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