Cusanovich, M. A., & Tollin, G. (1980) *Biochemistry 19*, 3343–3347.
Errede, B., & Kamen, M. D. (1978) *Biochemistry 17*, 1015–1027.
Frost, A. A., & Pearson, R. G. (1961) *Kinetics and Mechanism*, 2nd ed., Chapter 8, Wiley, New York.
Gibson, Q. H., Greenwood, C., Wharton, D. C., & Palmer, G. (1965) *J. Biol. Chem. 240*, 888–894.
Guzzo, A. V., & Tollin, G. (1963) *Arch. Biochem. Biophys. 103*, 231–243.
Hill, B. C., & Nicholls, P. (1980) *Biochem. J. 187*, 809–818.
Leigh, J. S., Jr., Wilson, D. F., Owen, C. S., & King, T. E. (1974) *Arch. Biochem. Biophys. 160*, 476–486.
Malmström, B. G. (1979) *Biochim. Biophys. Acta 549*, 281–303.
Petersen, L. C., & Cox, R. P. (1980) *Eur. J. Biochem. 105*, 321–327.
Shiga, K., & Tollin, G. (1976) in *Flavins and Flavoproteins* (Singer, T. P., Ed.) pp 422–433, Elsevier, Amsterdam.
Sutin, N., & Yandell, J. K. (1972) *J. Biol. Chem. 247*, 6932–6936.
Tiesjema, R. H., Muijsers, A. O., & van Gelder, B. F. (1973) *Biochim. Biophys. Acta 305*, 19–28.
Tollin, G., Chan, R. L., Malefyt, T. R., & Bruice, T. C. (1979) *Photochem. Photobiol. 29*, 233–243.
Wilson, D. F., Lindsay, J. G., & Brocklehurst, E. S. (1972) *Biochim. Biophys. Acta 256*, 277–286.
Yonetani, T. (1960) *J. Biol. Chem. 235*, 845–852.

Escherichia coli Phosphoenolpyruvate-Dependent Phosphotransferase System: Role of Divalent Metals in the Dimerization and Phosphorylation of Enzyme I†

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ABSTRACT: The function of divalent metal ions (Mg^{2+} and Mn^{2+}) in the dimerization and phosphorylation of enzyme I has been studied. Only a dimeric form of the enzyme can be phosphorylated [Misset, O., Brouwer, M., & Robillard, G. T. (1980) *Biochemistry 19*, 883–890; Hoving, H., Lolkema, J. S., & Robillard, G. T. (1981) *Biochemistry 20*, 87–93]. Kinetic studies of phosphoryl-group exchange between phosphoenolpyruvate and pyruvate and measurements of initial enzyme I phosphorylation rates revealed that a divalent metal ion must be bound to the enzyme to render the dimer active. Mn^{2+} binding experiments by means of electron paramagnetic resonance showed binding of at least one Mn^{2+} per unphosphorylated dimer with a binding constant comparable to the activation constant found in the kinetic studies and a 10-fold tighter binding of only one Mn^{2+} per phosphorylated dimer. Gel filtration experiments provided evidence that divalent metals produce about a 10-fold stabilization of the dimers, in addition to their effect on the specific dimer activity. The stability of the dimer was also strongly dependent on salts such as LiCl, NaCl, KCl, and a series of tetraalkylammonium chlorides. The relative effects of these salts suggest that hydrophobic interactions possibly play a significant role in enzyme I dimerization.

Enzyme I, a component of the bacterial PEP[1]-dependent phosphotransferase system, catalyzes the transfer of a phosphoryl group from PEP to a phospho-carrier protein HPr. This is the first step in a process that ultimately leads to the phosphorylation and concomitant transport of PTS sugars into the bacterial cell (Roseman, 1969; Postma & Roseman, 1976; Hengstenberg, 1977; Saier, 1977; Hays, 1978). During the last few years increasing evidence has been presented that the enzyme I catalyzed reaction proceeds via a phosphoenzyme I intermediate (Stein et al., 1974; Waygood & Steeves, 1980; Saier et al., 1980; Hoving et al., 1981). It has also been shown that the active dimeric form of the enzyme reversibly dissociates into inactive monomers (Waygood et al., 1979; Misset et al., 1980):

$$2E_I^m \rightleftharpoons E_I^d \quad (1a)$$

$$PEP + E_I^d \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_I^dP + Pyr \quad (1b)$$

$$E_I^dP + HPr \rightleftharpoons PHPr + E_I^d \quad (1c)$$

In the following paper (Misset & Robillard, 1982) data are presented indicating that the reactions in the above sequence, although basically correct, are a simplification because there is also an interaction between HPr and unphosphorylated enzyme I. It is also shown in the following paper that only one phosphoryl group can be bound per enzyme I dimer.

The phosphorylation of enzyme I is dependent on divalent metals, a property that this enzyme has in common with many other phosphoryl-group transferring enzymes. In a recently published study of enzyme I catalyzed phosphoryl-group exchange between PEP and pyruvate, we showed that Mg^{2+} shifted the monomer–dimer equilibrium of enzyme I toward the dimer side (Hoving et al., 1981). In a similar study Saier et al. (1980) demonstrated that Mn^{2+} and Co^{2+} can substitute for Mg^{2+}.

The purpose of the study presented here was to provide more insight into the role of divalent metal ions in enzyme I phosphorylation. Since phosphorylation can only proceed in

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[1] Abbreviations: PTS, phosphotransferase system; PEP, phosphoenolpyruvate; Pyr, pyruvate; DTT, dithiothreitol; TMA^+, tetramethylammonium; TEA^+, tetraethylammonium; TBA^+, tetrabutylammonium; EDTA, ethylenediaminetetraacetate; EPR, electron paramagnetic resonance; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/82/0421-3128$01.25/0

the presence of these ions, detailed information concerning their function is essential for an accurate understanding of the molecular mechanism of phosphoryl-group transfer.

In the experiments reported here Mn^{2+} was used because the binding of this ion to enzyme I and PEP can be determined by means of EPR. In addition to these binding studies, kinetic experiments including phosphoryl-group exchange and initial enzyme I phosphorylation measurements will be presented. Kinetic data obtained with Mn^{2+} and Mg^{2+} will be compared since the latter metal is commonly used in PTS assays and in bacterial growth media. Gel filtration data relevant to the monomer–dimer equilibrium of enzyme I will be reported. Since part of the effect of divalent metals on enzyme I activity appeared to involve stabilization of the dimeric form of the enzyme, attention will be paid to salts other than divalent metal chlorides that also affect the degree of dimerization.

Materials and Methods

Chemicals. PEP (monocyclohexylammonium salt) and pyruvate (sodium salt) were purchased from Sigma Chemical Co. [1-^{14}C]PEP (monocyclohexylammonium salt; sp act. 12 mCi/mmol) was purchased from Amersham.

Enzyme I. Enzyme I was purified from *Escherichia coli* P650 by the method of Robillard and co-workers based on a combination of both hydrophobic interaction chromatography (Robillard et al., 1979) and ion-exchange chromatography [see Misset & Robillard (1982) and Brouwer et al. (1982)]. The procedure including ion-exchange chromatography has now been used for 1 year in our laboratory for reasons mentioned in the following paper. The enzyme thus purified appeared to have a higher specific activity in the phosphorylation reaction than enzyme I purified by the original procedure. Although most of the experiments were carried out in Tris buffers, the purified enzyme was stored in a phosphate buffer (25 mM sodium phosphate, pH 7.2, containing 1 mM sodium azide and DTT) at −20 °C. Sodium azide was included in all solutions to prevent bacterial growth. Storage in Tris buffers at −20 °C appeared to cause a significant loss of activity within a few days. The activity could be partly restored by prolonged incubation with Mg^{2+} (or Mn^{2+}) and PEP. When stored in phosphate buffers at −20 °C, the enzyme is stable for months. Enzyme I concentrations were determined by measuring the amount of ^{14}C-labeled pyruvate formed upon incubation of enzyme I with ^{14}C-labeled PEP [see Brouwer et al. (1982)]. Thus, the enzyme I concentrations as reported in this paper are always concentrations of available phosphoryl-group binding sites and correspond to dimer concentrations [see Misset & Robillard (1982)].

Phosphoryl-Group Exchange Experiments. Phosphoryl-group exchange between PEP and pyruvate was monitored with [1-^{14}C]PEP. The incorporation of this label into pyruvate was measured as described previously (Hoving et al., 1981). When fast phosphoryl-group exchange was being measured, the reaction was stopped by diluting samples in cold water containing excess EDTA. Measurement of rapid exchange reactions was also made feasible by using the whole course of the exchange process (until equilibrium) for the determination of the rate constants, instead of initial exchange velocities [see Hoving et al. (1981)]. This method allowed the determination of rate constants from phosphoryl-group exchange processes with a $t_{1/2}$ of less than 0.5 min. Measurement of such fast exchange reactions was sometimes necessary, because high enzyme I concentrations had to be employed in order to have a significant percentage of the dimeric form.

Initial Enzyme I Phosphorylation. Initial rates of enzyme I phosphorylation were determined by incubating enzyme I with [1-^{14}C]PEP and then measuring the rate of pyruvate formation as in the phosphoryl-group exchange experiments.

Mn^{2+} Binding Experiments. Mn^{2+} binding studies were performed with EPR to detect unbound Mn^{2+}. First-derivative peak amplitudes were used as a measure of free Mn^{2+}. The measurements were carried out on a Varian E4 spectrometer equipped with a variable temperature control unit. The temperature of the sample was held at 37 °C by immersing the 1-mm quartz capillary containing the sample in a 5-mm quartz tube filled with toluene at 37 °C. The sample capillary and the glass capillary pipets used were coated with a silicone surface by using a 2% solution of dimethyldichlorosilane in 1,1,1-trichloroethane (Repelcote, Hopkin & Williams, Chadwell Heath, England). This coating was necessary to prevent binding of protein and, especially, Mn^{2+} to glass and quartz. The enzyme preparations used in these experiments were freed of metals by extensive dialysis against a Tris buffer containing Chelex-100 (Bio-Rad).

Theory of Phosphoryl-Group Exchange

In the following sections experiments will be described in which the enzyme I catalyzed phosphoryl-group exchange between PEP and pyruvate was used to investigate the influence of divalent metals on the phosphorylation of enzyme I. Our theoretical approach to this exchange reaction has been detailed in a previous article (Hoving et al., 1981). For clarity, this treatment will be summarized here, with emphasis on those elements that are of particular importance for the experiments to be presented.

The full reaction scheme for the (de)phosphorylation of enzyme I is

$$E_I^d + PEP \underset{p_{-1}}{\overset{p_1}{\rightleftharpoons}} E_I^d\text{-}PEP \underset{r_2}{\overset{p_2}{\rightleftharpoons}} E_I^dP\text{-}Pyr \underset{r_1}{\overset{r_{-1}}{\rightleftharpoons}} E_I^dP + Pyr \quad (2)$$

$$K_D \Big\updownarrow$$

$$2E_I^m$$

The interconversion of PEP and pyruvate, or phosphoryl-group exchange, is measured at chemical equilibrium with ^{14}C as a label. When steady-state conditions apply for the kinetics of ^{14}C transfer, eq 2 reduces to eq 1a and 1b with

$$k_1 = \frac{p_1 p_2 r_{-1}}{(p_2 + p_{-1})(r_2 + r_{-1}) - r_2 p_2} \quad (3a)$$

$$k_{-1} = \frac{r_1 r_2 p_{-1}}{(p_2 + p_{-1})(r_2 + r_{-1}) - r_2 p_2} \quad (3b)$$

Since at chemical equilibrium the concentrations of E_I and E_IP are constant, the exchange reaction can be described as a pseudo-first-order process:

$$[^{14}C]PEP \underset{k_{-1}^*}{\overset{k_1^*}{\rightleftharpoons}} [^{14}C]Pyr \quad (4)$$

$$k_1^* = k_1[E_I^d] \qquad k_{-1}^* = k_{-1}[E_I^dP] \quad (5)$$

These first-order rate constants can be obtained from the time course of an exchange reaction starting with ^{14}C-labeled PEP and unlabeled pyruvate.

Since the active enzyme I dimer reversibly dissociates into inactive monomers, an accurate study of the role of M^{2+} in the reaction requires experiments with varying total enzyme concentrations at each $[M^{2+}]$. When the influence of M^{2+} on the exchange reaction is measured at only one enzyme concentration, it will be impossible to decide whether this influence is due to a changing specific activity of dimeric enzyme I or

to a changing degree of dimerization or both. If, however, several enzyme concentrations are employed, the kinetic data can be treated in such a way as to reveal the effect of M^{2+} on both features separately [see also Kurganov (1967)]. This can be achieved by plotting the data according to eq 7, which can be derived from reaction eq 1 and 2. The total enzyme concentration is

$$[E_I]^{tot} = [E_I^d] + [E_I^dP] + [E_I^d\text{–}PEP] + [E_I^dP\text{–}Pyr] + (1/2)[E_I^m] \quad (6)$$

By use of chemical equilibrium relations, this equation can be rewritten as

$$\frac{[E_I]^{tot}}{k_1^*} = \frac{1}{k_1} + \frac{1}{k_{-1}}\frac{[PEP]}{[Pyr]} + [PEP]\left(\frac{1}{k_1}\frac{p_1}{p_{-1}} + \frac{1}{k_{-1}}\frac{r_1}{r_{-1}}\right) + \frac{1}{2}\left(\frac{K_D}{k_1}\right)^{1/2}(k_1^*)^{-1/2} \quad (7)$$

The first term in the right-hand side of eq 7 corresponds to the first term in the right-hand side of eq 6 etc. When k_1^* is measured at different total enzyme concentrations and $[E_I]^{tot}/k_1^*$ is plotted vs. $(k_1^*)^{-1/2}$, a straight line is obtained, as shown in Figure 1. This straight line provides a means to extrapolate the data to 100% dimeric enzyme I. As can be seen from a comparison of eq 6 and 7, the intercept represents the value of $[E_I]^{tot}/k_1^*$ at 100% dimeric enzyme I, which is, therefore, the reciprocal value of the specific activity of dimeric enzyme I. At any point on the line the relative values of the intercept and of the ordinate minus intercept represent the relative amounts of dimeric and monomeric enzyme I, respectively. At low total enzyme concentrations, almost all of the enzyme will exist in the monomeric form; the last term in eq 7 will then be the dominating one, and k_1^* will increase quadratically with $[E_I]^{tot}$. The value of k_1 can, in principle, be obtained from the intercept by using low enough [PEP] and high enough [Pyr] (compare the first four terms on the right-hand side of eq 7). When k_1 is known, the value for K_D can be obtained from the slope (see the last term on the right-hand side of eq 7).

Results

Phosphoryl-Group Exchange Experiments. In this section experiments will be presented in which the phosphoryl-group exchange method, as described in the previous two sections, was used to probe the influence of Mn^{2+} and Mg^{2+} on the phosphorylation of enzyme I. The focus is mainly on Mn^{2+}, because the binding of this ion to the enzyme and to the substrate can be measured by means of EPR, thus allowing a comparison of kinetic data with binding constants.

In order to account for the monomer–dimer equilibrium of enzyme I, exchange experiments were carried out at several total enzyme concentrations for each $[Mn^{2+}]$. The data were plotted according to eq 7, and the result is shown in Figure 1. As can be seen from this figure, increasing $[Mn^{2+}]$ stimulates the exchange reaction up to a concentration of about 600 μM. At low $[Mn^{2+}]$ the slopes of the lines are proportional to $[Mn^{2+}]^{-1/2}$, which means that at low Mn^{2+} and enzyme I concentrations the exchange rate is proportional to $[Mn^{2+}]$. This can be seen from the last term in eq 7. This behavior persists with $[Mn^{2+}]$ below 10 μM until, in the absence of Mn^{2+}, no exchange is measured at all. Thus, at low Mn^{2+} and enzyme I concentrations, the exchange rate increases quadratically with $[E_I]^{tot}$ and linearly with $[Mn^{2+}]$, indicating that two enzyme I monomers and one Mn^{2+} are required for the

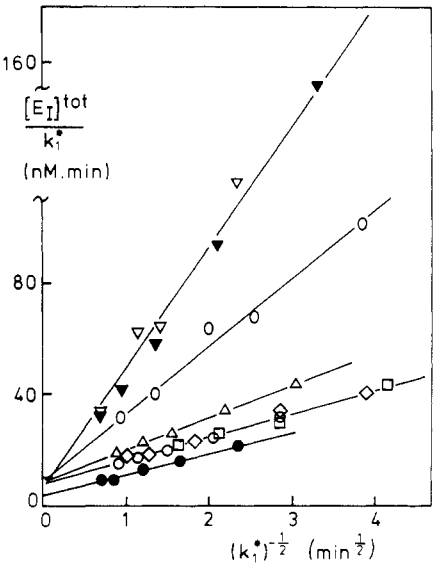

FIGURE 1: Forward first-order rate constant of phosphoryl exchange between 900 μM pyruvate and 7.5 μM PEP (open symbols) or 3.75 μM PEP (closed symbols), as a function of total enzyme I concentration and at different $[Mn^{2+}]$: 10 (▽), 40 (0), 160 (△), 640 (○), 1280 (□), and 5120 μM (◇). Experimental conditions: 50 mM Tris, 50 mM NaCl, 1 mM NaN_3, and 1 mM DTT, pH 7.2, at 37 °C.

rate-determining steps in the reaction. Similar results were obtained in experiments with Mg^{2+}, except that concentrations of this ion about 35 times higher than that of Mn^{2+} were required for the same exchange rates.

The next step is to determine whether this requisite metal ion binds to enzyme I or to PEP. This can be done by determining, from the kinetic experiments, the K_D value of the Mn^{2+} complex responsible for the activation and comparing this with the K_D values of the Mn–PEP and Mn–enzyme I complexes. This is only possible if the reaction is not saturated with respect to PEP. Therefore, this point deserves some attention first. In the case of saturation with PEP, $[E_I]^{tot}/k_1^*$ will be proportional to [PEP], and a possible influence of Mn^{2+} on k_1 can be masked because the [PEP]-dependent terms in eq 7 determine k_1^*. Experiments at two PEP concentrations were carried out with 640 and 10 μM Mn^{2+} (compare the open and closed symbols in Figure 1). The two intercepts at 640 μM Mn^{2+} show that at 100% dimeric enzyme I the exchange reaction is saturated with respect to PEP and, therefore, that all the dimeric enzyme must either be phosphorylated or have bound PEP (compare eq 6 and 7). For this reason the intercepts cannot be used to estimate a dissociation constant for a possible Mn^{2+}–enzyme I or Mn^{2+}–PEP complex responsible for enzyme I phosphorylation. The slopes in Figure 1 can, however, be used for this purpose because they appear to be independent of [PEP]. In fact, this behavior proves that the enzyme I monomers do not bind PEP to any significant extent, because it is the omission of this binding in reaction scheme 2 that makes the slopes as predicted by eq 7 independent of [PEP]. Using the slopes in Figure 1, we can immediately conclude that the dissociation constant of Mn^{2+} from the Mn^{2+}-activated complex must be well below 600 μM (later in this paper evidence will be presented that this dissociation constant must be even well below 160 μM). This result is not compatible with the Mn^{2+}–PEP complex as the requisite substrate form, because the dissociation constant of this complex is about 2 mM (Wold & Ballou, 1957; Midvan & Cohn, 1966). Therefore, the requisite metal ion must be bound to enzyme I. The Mn^{2+} binding data (see below) show a binding of Mn^{2+} to enzyme I, which can account for the observed effect

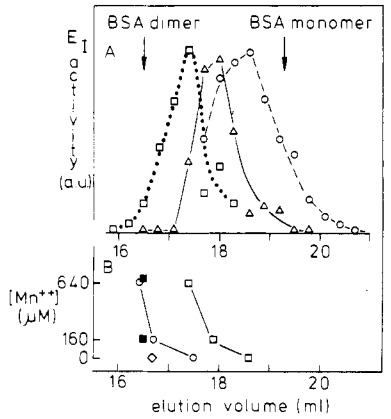

FIGURE 2: (A) Elution profiles of enzyme I on a Sephacryl S200 column, in the presence of different concentrations of Mn^{2+}: 0 (---), 160 (—), and 640 μM (···). Experimental conditions: 50 mM Tris, 50 mM NaCl, 1 mM NaN_3, and 1 mM DTT, pH 7.2, at 37 °C. 200-μL 0.5 μM enzyme I samples were applied to the column: l = 100 cm; ϕ = 0.7 cm; elution rate = 1.7 mL/h. (B) Elution position of enzyme I peak maximum as a function of $[Mn^{2+}]$, at different enzyme I concentrations: 12.5 (◇), 2.5 (○), and 0.5 μM (□), in the absence (open symbols) and presence (closed symbols) of 1 mM PEP. The symbols in (A) do not correspond with those in (B).

of Mn^{2+} in the kinetic studies. We wish to emphasize that no inhibition by Mn^{2+} was observed at concentrations up to 5 mM, in contrast with data presented by Saier et al. (1980).[2]

Anticipating the Mn^{2+} binding results, we can conclude that the requisite divalent metal ion binds to enzyme I, and the next question is whether it is required for the dimerization of the enzyme or whether it is required for the activity of already existing dimers. The data in Figure 1 point out that at increasing $[Mn^{2+}]$ the monomer–dimer equilibrium is shifted to the dimer side, whereas the specific dimer activity (intercept) does not noticeably change. Since the dimers appear to be either phosphorylated or complexed with PEP under the experimental conditions used, it cannot be excluded that k_1 is affected by Mn^{2+}. Therefore, the Mn^{2+} dependency of the slopes can still be brought about via k_1 instead of K_D. It should be noticed that the observed shifting of the monomer–dimer equilibrium concerns the equilibrium between free monomers and phosphorylated or PEP-complexed dimers and could therefore be caused not only via K_D but also via k_1.

In order to discriminate between the two possible mechanisms, experiments were designed to study the influence of divalent metals on K_D and k_1 separately. These experiments, including measurements of initial rates of enzyme I phosphorylation and gel filtration, will be presented in the following sections.

Gel Filtration. The kinetic data presented in the previous section provide evidence that a divalent metal ion is required for activity of enzyme I. It appeared that the monomer–dimer equilibrium is shifted to the dimer side by Mn^{2+} in a situation where the dimers are saturated with PEP or phosphorylated. We will now show, by gel filtration data, to what extent Mn^{2+} affects the equilibrium between monomers and free dimers of enzyme I. Enzyme I samples (200 μL) were applied to a 100 × 0.7 cm Sephacryl S200 column run at 1.7 mL/h. Enzyme I activity in the eluate was determined according to Robillard et al. (1979). The experimental conditions during the column run were the same as in the phosphoryl-group exchange experiments, except that PEP and pyruvate were now absent. The results are summarized in Figure 2. The elution positions of bovine serum albumin monomers and dimers, which have the same molecular weights as the enzyme I monomers and dimers, are indicated in the figure. In the upper part of Figure 2 elution profiles of enzyme I in the presence of 0, 160, and 640 μM $MnCl_2$ are shown. The shifting of the enzyme I peak shows that, indeed, Mn^{2+} gives rise to an increasing dimerization of the enzyme. Similar results have been obtained with Mg^{2+}. A striking result is that enzyme I elutes in front of the monomer position in the absence of divalent metals, indicating that even if no divalent metal is present there still is a certain degree of dimerization. This is confirmed by an experiment in which the enzyme I concentration was varied, in the absence of Mn^{2+} (see Figure 2B). Phosphorylation results in a marked stabilization of the dimers (closed symbols in Figure 2B), in accordance with data reported by Misset et al. (1980).

In principle, it should be possible to determine the relative amounts of monomeric and dimeric enzyme I from data such as given in Figure 2B. However, a reasonably large error is inherent in calculations of K_D values from such data, because of the peak widths and the dilution during elution. Furthermore, the hydrophobic nature of enzyme I (Robillard et al., 1979) might introduce additional errors at high enzyme I concentrations. The determination of changes in K_D upon the addition of Mn^{2+} at constant enzyme concentration is more accurate, because only differences in the dilution during elution are of importance. It can be inferred from the data in Figure 2B that the addition of 640 μM Mn^{2+} produces approximately a 10-fold decrease of the dissociation constant of dimeric enzyme I.[3] This change in K_D can account for the observed stimulation of the phosphoryl-group exchange rates at high $[Mn^{2+}]$ ($\geq$160 μM; see Figure 1). At much lower $[Mn^{2+}]$, however, there is only a small effect on the dimerization whereas the phosphoryl-group exchange rate remains proportional to $[Mn^{2+}]$. It must therefore be concluded that, although necessary, dimeric enzyme I is not sufficient for activity: the enzyme does dimerize in the absence of divalent metals, but the dimer is only active if it has bound at least one Mn^{2+}, with a K_D well below 160 μM.

Initial Rate of Enzyme I Phosphorylation. So far, the combination of both kinetic and gel filtration data leads to the conclusion that at low $[Mn^{2+}]$ there must be a linear dependence of the specific activity of dimeric enzyme I on $[Mn^{2+}]$. This effect was not found directly in the phosphoryl-group exchange experiments, presumably because dimeric enzyme I was saturated with PEP or phosphorylated. As can be seen from Figure 1, at much lower PEP concentrations the amount of dimeric enzyme would have been negligibly small (see also the gel filtration results). Yet, on the basis of the phospho-

[2] Saier et al. (1980) reported a strong inhibition of enzyme I activity by millimolar Mn^{2+} concentrations in phosphoryl-group exchange experiments with 1-h incubations. In our phosphoryl-group exchange experiments the time courses were only a few minutes long, and no inhibition was found. Upon prolonged exposure to millimolar Mn^{2+} concentrations we also found a decrease of enzyme I activity.

[3] There seems to be some confusion in the literature concerning the accuracy of a K_D determination by using this method (Valdes & Ackers, 1979). Zimmerman & Ackers (1971) have shown that the K_D calculated from a computer-simulated column run of a solute exhibiting a monomer–tetramer equilibrium may be a factor of 10^5 too high. In their calculation, however, they did not account for dilution during the column run. A 50-fold dilution (the simulated situation was a 20-μL sample applied to a 12 × 1 cm column, and the simulations themselves showed a dilution in this order) would explain the factor 10^5 because the concentration appears to the third power in the calculated K_D for a monomer–tetramer equilibrium. For a monomer–dimer equilibrium this problem is far less serious because the calculated K_D is only proportional to the concentration. In our experiments the dilution at the peak maxima is only about 7-fold, and only the differences in this factor affect the calculated change in K_D upon addition of Mn^{2+}.

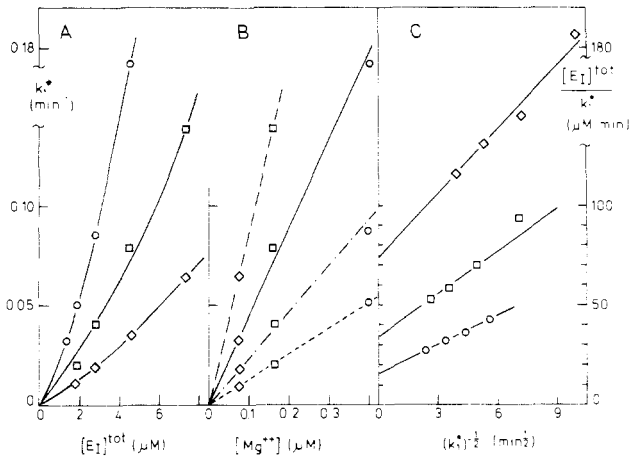

FIGURE 3: (A) Pseudo-first-order rate constant of the reaction of excess enzyme I with 0.25 μM PEP as a function of total enzyme concentration, at different Mg^{2+} concentrations: 0.075 (◇), 0.17 (□), and 0.40 μM (○). Experimental conditions: 15 mM sodium phosphate, 1 mM NaN_3, and 1 mM DTT, pH 7.2, at 37 °C. (B) Same data as in (A) now plotted vs. $[Mg^{2+}]$ at different total enzyme I concentrations: 1.9 (---), 2.8 (-·-), 4.6 (—), and 7.4 μM (--). The symbols correspond with those in (A). (C) Same data as in (A) and (B) (corresponding symbols), replotted to account for the monomer–dimer equilibrium of enzyme I.

ryl-group exchange and gel filtration data it should be possible to measure enzyme I phosphorylation at enzyme concentrations high enough to yield a substantial amount of free dimers (micromolar concentrations) and at divalent metal concentrations low enough to render the reaction rate sufficiently slow to be measured accurately. The high enzyme I concentrations to be employed in such experiments should allow the measurement of initial phosphorylation rates without the need for unlabeled pyruvate as a phosphoryl-group acceptor. Therefore, experiments monitoring the initial rate of enzyme I phosphorylation at varying and extremely low divalent metal concentrations were carried out in an attempt to measure the supposed influence of M^{2+} on the specific activity of dimeric enzyme I.

It appeared that even the traces of metal present in the chemicals and enzyme preparation used caused a fast phosphorylation of enzyme I. In order to circumvent this problem of a high background phosphorylation rate, all experiments were carried out in the presence of 200 μM EDTA to which varying amounts of $MgCl_2$ were then added. The free Mg^{2+} concentration can be calculated from the known binding constants of Mg^{2+} and H^+ to $EDTA^{4-}$ and phosphate (Martell, 1964). Although the values thus calculated may be somewhat inaccurate, the variation of $[Mg^{2+}]^{free}$ with the total added Mg^{2+} concentration can be calculated accurately as long as the EDTA is not saturated with metal. These experiments could not be performed with Mn^{2+} because it binds too strongly to EDTA to allow such a titration. In view of the similarity of the phosphoryl-group exchange data obtained with Mn^{2+} and Mg^{2+}, the substitution of Mg^{2+} for Mn^{2+} seems justified. The experiments were performed with a PEP concentration at least 5 times lower than the total enzyme concentration. As a result, the concentration of unphosphorylated enzyme remained nearly constant during the conversion of the total amount of PEP into pyruvate, causing an exponential decay of the PEP concentration. The first-order rate constant of this process was measured at varying total enzyme and Mg^{2+} concentrations. The plots in Figure 3A exhibit the characteristic increase of this rate constant with the total enzyme concentration, indicating that there is no saturation in this case. Since the concentration of PEP is much lower than the total enzyme concentration, saturation does not mean that all the enzyme has bound substrate but that all the substrate is in the complexed form. As a result, in case of saturation, the reaction rate should remain constant at increasing enzyme concentrations rather than at increasing substrate concentrations. The fact that no saturation has occurred plus the exponential decay of the PEP concentration means that the steady-state condition applies. Therefore, the pseudo-first-order rate constant of the reaction is $k_1[E_I]$ with the same k_1 as in the phosphoryl-group exchange experiments.

The data in Figure 3A are replotted in Figure 3B, showing a linear relationship between the phosphorylation rate and $[Mg^{2+}]$. Figure 3A,B, obtained from the most direct experiment possible, proves once more that two monomers of enyzme I and one divalent metal ion are required in the rate-determining steps of enzyme I phosphorylation. In Figures 3C the data are replotted in such a way as to allow the extrapolation to 100% dimeric enzyme I. From the intercepts (now representing $1/k_1$) it can be seen that the specific phosphorylation rate of dimeric enzyme I (k_1) is proportional to $[Mg^{2+}]$. The linear increment of the phosphorylation rate with $[Mg^{2+}]$ depicted in Figure 3B can thus be ascribed to an increasing specific reaction rate of dimeric enzyme I, whereas the degree of dimerization is not altered. This result is in agreement with the phosphoryl-group exchange and gel filtration data and confirms the conclusion drawn from the combination of these data, as mentioned in the beginning of this section. From the slopes $[(1/2)(K_D/k_1)^{1/2}]$ and intercepts $(1/k_1)$ in Figure 3C we have calculated a dissociation constant of 6 μM for dimeric enzyme I at all three Mg^{2+} concentrations employed. This is essentially the K_D value of enzyme I dimers that have not bound divalent metal, in 15 mM sodium phosphate at 37 °C. As will be subsequently shown, this value depends strongly on the salt concentration.

The data now available can be summarized as follows: (i) only dimeric enzyme I can become phosphorylated (phosphoryl-group exchange and initial phosphorylation data), (ii) the enzyme does dimerize in the absence of divalent metals (gel filtration and initial phosphorylation data), but (iii) a divalent metal ion must be bound to bring the dimer into its active form (initial phosphorylation data). In addition to this there is a stabilizing effect of divalent metals on the enzyme I dimers (gel filtration data).

Mn^{2+} Binding. Using EPR to detect unbound Mn^{2+}, we found a value of 2.2 mM for the dissocation constant of the Mn^{2+}–PEP complex, which is in reasonable agreement with the values reported in the literature: 1.8 mM (Wold & Ballou, 1957) and 1.9 mM (Mildvan & Cohn, 1966). From a comparison of these values with the kinetic data reported above we have already concluded that a binding of Mn^{2+} to enzyme I ($K_D < 160$ μM) rather than to PEP is required for enzyme I phosphorylation. EPR experiments were then performed to see if a binding of Mn^{2+} to enzyme I could be detected, which would account for kinetic data.

Figure 4 represents a Hughes–Klotz plot of a titration of enzyme I with Mn^{2+}, carried out under the same conditions as the phosphoryl-group exchange experiments. If only one type of metal binding site is present on the enzyme, the data should lie on a straight line:

$$[E_I]^{tot}/[Mn^{2+}]^{bound} = (K_D/n)(1/[Mn^{2+}]^{free}) + 1/n \quad (8)$$

In this equation K_D represents the dissociation constant of the enzyme–metal complex. Since the total enzyme concentration is expressed as a concentration of available phosphoryl-group binding sites, corresponding to a concentration of dimers, n represents the number of Mn^{2+} binding sites per dimer.

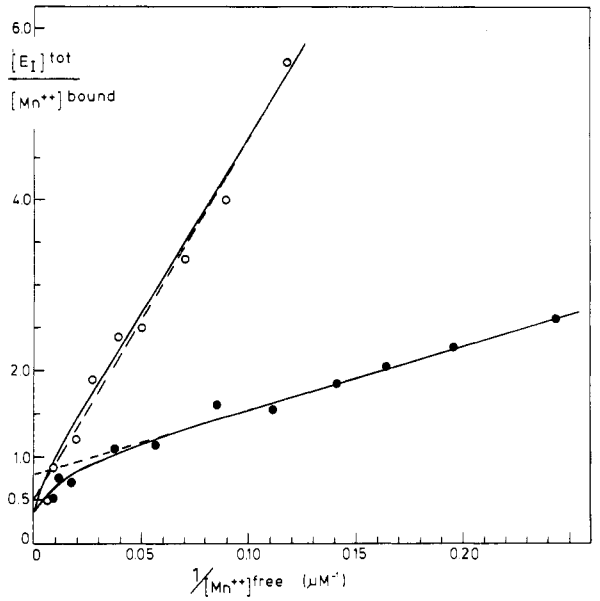

FIGURE 4: Hughes–Klotz plot representing the binding of Mn^{2+} to 8.3 μM enzyme I (open symbols) and to 7.1 μM phosphoenzyme I (closed symbols), as detected by means of EPR. Experimental conditions: 50 mM Tris, 50 mM NaCl, and 1 mM DTT, pH 7.2, at 37 °C (open symbols), and the same plus 100 μM PEP (closed symbols).

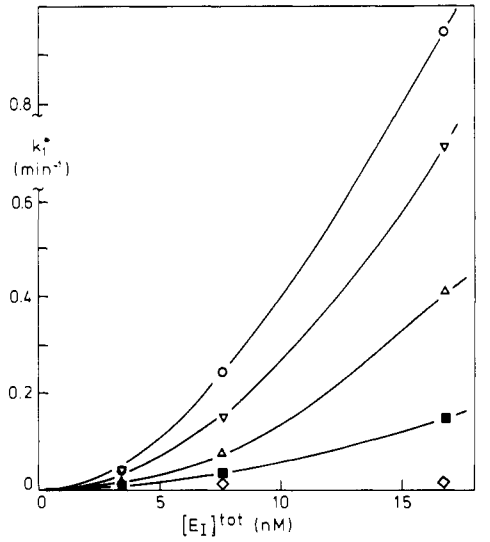

FIGURE 5: Forward first-order rate constant of phosphoryl exchange between 900 μM pyruvate and 7.5 μM PEP as a function of total enzyme I concentration. Experimental conditions: 10 mM Tris, 1 mM NaN_3, and 1 mM DTT, pH 7.2, at 37 °C. (Closed symbols) No additons; (open symbols) plus 100 mM NaCl (○), TMACl (▽), TEACl (△), and TBACl (◇).

At first sight the results obtained with unphosphorylated enzyme I (open symbols) point to two binding sites per dimer with K_D = 85 μM (---). However, at the rather high Mn^{2+} concentrations required to determine the number of binding sites there may be some weaker binding as well. This makes the number of specific binding sites, and hence the corresponding K_D value, somewhat imprecise. For example, two sites with K_D = 500 μM plus one site with K_D = 48 μM also give an excellent fit (—). Whichever fit is chosen, however, there must be at least one binding site per dimer with a K_D in the 40–100 μM range, which is in good agreement with the kinetic data.

Upon phosphorylation the enzyme exhibits a striking change in its Mn^{2+} binding properties (closed symbols in Figure 4). The data can be accounted for by one binding site with K_D = 7.5 μM plus two sites with K_D = 250 μM (—). Since the binding of Mn^{2+} to phosphorylated enzyme I is much stronger than to the unphosphorylated enzyme, the number of specific binding sites and the corresponding K_D value can be determined much more accurately. In fact, one tight binding site with K_D = 7.5 μM produces the only reasonable fit possible, as can be seen from the extrapolation of the tight binding in Figure 4 (---). The extrapolation yields a number of sites smaller than two, and this value can only decrease when the data in the right side of the figure are corrected for the contribution of the weaker binding sites. Therefore it can be concluded that one Mn^{2+} ion is tightly bound per phosphorylated dimer or, since the enzyme concentration was actually determined as a concentration of phosphorylated sites, that one Mn^{2+} is tightly bound per phosphoryl group bound. This result suggests that it is the one divalent metal ion required for enzyme I phosphorylation that becomes approximately 10 times more tightly bound upon phosphorylation.

Finally, attempts were made to determine the binding constants of Mg^{2+} to enzyme I and phosphoenzyme I via the displacement of bound Mn^{2+} from the enzyme by Mg^{2+}. It appeared that about a 50-fold excess of Mg^{2+} over Mn^{2+} is necessary to displace 50% of the bound Mn^{2+} from the unphosphorylated enzyme. This is comparable with the results from kinetic studies, in which it was found that Mg^{2+} concentrations about 35-fold higher than $[Mn^{2+}]$ were required to get the same reaction rates. The difference in binding constants between Mn^{2+} and Mg^{2+} for the phosphorylated enzyme seemed to be even more than a factor of 50.

Effect of Salts. During the execution of the kinetic experiments it appeared that the addition of salts other than divalent metal chlorides also strongly stimulated the phosphorylation rate. For example, the addition of 100 mM NaCl to a 10 mM Tris buffer caused a 6-fold increase in the phosphorylation rate at low enzyme I concentrations. A stimulation by salts was also observed in experiments with Tris-HCl, sodium phosphate, and potassium, sodium, lithium, and tetramethylammonium chlorides, suggesting that it is a general salt effect instead of a specific ion requirement that is responsible for the enhancement of the phosphorylation rate.

A possible influence of salts on the stability of the active conformation of enzymes can be investigated by studying the effect of a lyotropic series on enzymatic activity (Von Hippel & Schleich, 1969; Record et al., 1978). The anions, ranging from SCN^- as a strong destabilizer to SO_4^{2-} as a strong stabilizer, could not be used in our case because they bind divalent metals to different extents and would, in that way, affect the measured enzyme I activity. Therefore, a series of tetraalkylammonium chlorides and monovalent metal chlorides was employed in phosphoryl-group exchange experiments. The effect of 100 mM tetraalkylammonium chloride and sodium chloride, when added to a 10 mM Tris buffer, is shown in Figure 5. The addition of 100 mM lithium or potassium chloride resulted in about the same exchange rates as 100 mM sodium chloride (not shown). Although the relative effects of the monovalent metal chlorides do not correspond with a lyotropic series, the observed effects of the tetraalkylammonium chlorides do. The latter effects, especially the strong inhibition by the tetrabutylammonium ion, suggest that a (de)stabilization of the active conformation of the enzyme is involved. Lyotropic effects on the stability of native protein conformations usually occur in the molar salt concentration range [see Von Hippel & Schleich (1969) and reference cited therein]. Here we found pronounced effects at much lower salt concentrations. The high sensitivity of the enzymatic

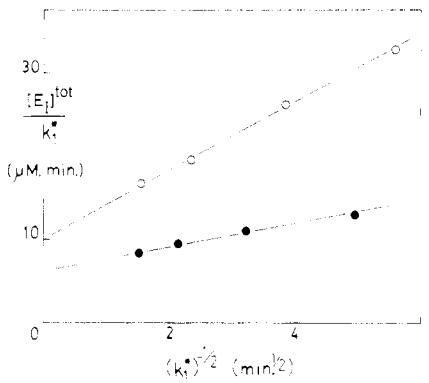

FIGURE 6: Pseudo-first-order rate constant of the reaction of excess enzyme I with PEP, as a function of total enzyme concentration. Experimental conditions: 15 mM sodium phosphate, 1 mM NaN_3, and 1 mM DTT, pH 7.2, at 37 °C (open symbols), and the same plus 50 mM NaCl (closed symbols).

activity to low concentrations of these salts may arise from an effect of salt concentration on the stability of the dimeric form of enzyme I.

This possibility was investigated by repeating a few of the gel filtration runs described earlier in this paper with TBACl instead of NaCl, because TBACl should cause a significant dissociation of dimeric enzyme I. In the presence of 640 μM $MnCl_2$ and 50 mM NaCl, 2.5 and 0.5 μM enzyme I exhibited a high degree of dimerization (see Figure 2B). When the NaCl was replaced by TBACl, both peaks appeared to be shifted to the monomer side by about 1.2 mL, indicating an increase in the dissociation constant of approximately a factor of 10. This effect on K_D could easily explain the difference in enzyme I activity between the experiments with NaCl and TBACl depicted in Figure 5. As shown earlier in this paper, the kinetic data themselves, if plotted properly, can show whether the dissociation constant of dimeric enzyme I is affected. From the foregoing sections it is clear that phosphoryl-group exchange data are not suitable for this purpose and that measurements of initial enzyme I phosphorylation rates are required to determine K_D. The effect of 50 mM NaCl on the initial enzyme I phosphorylation rate in 15 mM sodium phosphate is shown in Figure 6. The dissociation constant of dimeric enzyme I decreases by a factor of 3.5 upon addition of 50 mM NaCl. There is also an almost 2-fold increase in the specific dimer activity, which is not surprising for an enzyme that binds a strongly negatively charged substrate. Thus, both gel filtration and kinetic data indicate that the marked effect of neutral salts on enzyme I activity is mainly brought about via the stability of the enzyme I dimers.

Discussion

Specific Activity of Enzyme I. As stated under Materials and Methods, enzyme I isolated by our modified procedure exhibits a higher specific activity than the enzyme purified by the original procedure. This increase in the specific activity can be directly observed from a comparison of the intercepts in Figure 1 with Figure 4B of our previous article (Hoving et al., 1981). From this comparison it appears that the dimerization properties have changed as well. It seems that the enzyme as originally purified could be brought into its dimeric form more easily than the preparation we have now but that those dimers were less active. The forward rate constant of enzyme I phosphorylation was found to be 3×10^7 M^{-1} min^{-1} for the original preparation. Inspection of Figure 1 and eq 6 reveals that $1/k_1$ must now be much smaller than 5 nM min, or $k_1 \gg 2 \times 10^8$ M^{-1} min^{-1}. A value of approximately 1×10^9 M^{-1} min^{-1} for k_1 was obtained from the slope of a $[E_I]^{tot}/k_1^*$ vs. $(k_1^*)^{-1/2}$ plot of phosphoryl-group exchange results in 15 mM sodium phosphate at a saturating level of Mg^{2+} by using a value of 0.6 μM for the K_D of dimeric enzyme I under these conditions. This latter value was estimated from a 10-fold decrease of K_D (6 μM in the absence of divalent metals) by a saturating amount of divalent metals. A comparable value for k_1 was found in experiments monitoring the steady-state rate of sugar phosphorylation (Misset, 1981).

Role of M^{2+} in Enzyme I Phosphorylation. The main conclusions from the studies concerning the function of divalent metals in enzyme I phosphorylation have already been mentioned under Results and will only be briefly summarized here. Some remaining questions will then be discussed. In the following, results obtained with both Mn^{2+} and Mg^{2+} will be discussed. Since qualitatively the same results were obtained with Mg^{2+} and Mn^{2+}, the metal ion will be denoted by the symbol M^{2+}, unless reference is made to a specific metal.

The stimulatory effect of M^{2+} in the phosphoryl exchange experiments could be attributed to a specific binding to the enzyme. Binding of M^{2+} to the substrate, as seems to be required for ATP in kinase-catalyzed reactions (Knowles, 1980), is not required here. From the initial enzyme I phosphorylation data it could be inferred (i) that only dimeric enzyme I can be phosphorylated [see also Hoving et al. (1981)], (ii) that the enzyme dimerizes without M^{2+}, but (iii) that one M^{2+} must be bound per dimer to render it active. These conclusions are confirmed by the phosphoryl-group exchange and gel filtration data. Although M^{2+} is not required for the dimerization of enzyme I, gel filtration studies revealed that M^{2+} does stabilize the dimers.

Data presented in the accompanying paper demonstrate that only one phosphoryl group is bound per enzyme I dimer. Here we have shown the requirement of one M^{2+} for enzyme I phosphorylation and also a tight binding of one M^{2+} per dimer upon phosphorylation. On the assumption that both monomers are identical, these data point to a half-of-the-sites reactivity for this enzyme. We cannot eliminate the possibility that both subunits bind a Mn^{2+} with a K_D corresponding with the kinetic data, but the kinetic data only give evidence for the involvement of one divalent metal ion in the phosphorylation of the enzyme.

A question that arises immediately is whether both activation and stabilization of the dimer are brought about by the same M^{2+}. The answer cannot be directly obtained from the data now available. A definite answer could be given if both features would depend on M^{2+} with equal binding constants and if only one such M^{2+} would bind to the enzyme or if both effects would be caused by M^{2+} with different binding constants. The initial phosphorylation data do not contain information concerning M^{2+} binding constants because of the low $[M^{2+}]$ used. In the phosphoryl-group exchange experiments the effects of M^{2+} on both dimer activity and dimer stability are present. Because of the saturation with PEP in these experiments, the two effects cannot be observed separately, and the corresponding metal binding constants cannot be determined. Therefore, the gel filtration data must also be used and, consequently, only approximate values of the Mn^{2+} binding constants corresponding with dimer activation and stabilization can be obtained. As pointed out under Results, the stimulation of the phosphoryl-group exchange rate by Mn^{2+} concentrations above 160 μM is only caused by an increasing degree of dimerization. Therefore, the stimulation of k_1 that occurs at low Mn^{2+} concentrations levels off at 160 μM Mn^{2+}. This means that the dissociation constant of the

enzyme–Mn^{2+} complex, responsible for dimer activity, is well below 160 μM. Unlike dimer activity, the dimer stability can only be affected by metal concentrations in the order of or higher than the dissociation constant of the corresponding metal–dimer complex. Since there is already a pronounced effect on the degree of dimerization at 160 μM Mn^{2+}, this dissociation constant must be well below 160 μM as well. Therefore, if only one Mn^{2+} would bind per dimer with a dissociation constant well below 160 μM, we could then conclude that the same Mn^{2+} is responsible for both the activity and stabilization of the dimer. However, there is some uncertainty from the EPR data as to whether one or two Mn^{2+} ions bind to the unphosphorylated dimer with a dissociation constant in this range. If two Mn^{2+} ions bind per dimer, it might still be possible that one is responsible for the activity of the dimer and that the other affects the dimer stability. A perfect symmetry of the dimer until one subunit is phosphorylated would rule out this possibility. In this case each subunit is expected to bind one M^{2+}, which would be required for the phosphorylation of the corresponding subunit and which would also be involved in the stabilization of the dimer.

We cannot, from our present data, propose a mechanism by which the essential M^{2+} activates the enzyme I dimer. Structural information and accurate data on the binding of PEP in the absence and presence of M^{2+} are necessary. In principle, K_m values for PEP could be obtained from kinetic experiments such as phosphoryl-group exchange. However, as can be seen from Figure 1, determination of K_m requires much lower PEP concentrations than were used here (compare open and closed symbols at 640 μM Mn^{2+}). Extrapolation of 100% dimeric enzyme I will then require higher total enzyme concentrations, rendering the exchange velocity too fast to be measured by standard techniques. Some authors have presented apparent K_m values for PEP (Waygood & Steeves, 1980; Saier et al., 1980), but since the kinetic experiments were only performed at one relatively low enzyme I concentration, these values must depend on the total enzyme concentration and are not applicable to this type of mechanistic consideration.

An aspect of the interaction of divalent metals with enzyme I which may also have implications for mechanistic studies is that they catalyze phosphoenzyme I hydrolysis. In the presence of 2.5 mM $MgCl_2$ at pH 7.2, for example, the rate constant for hydrolysis was found to be 0.05 min^{-1}, whereas under the conditions employed in the phosphorylation experiments as shown in Figure 3, no noticeable hydrolysis occurred [data not shown; the experimental procedure has been described by Brouwer et al. (1982)].

Effect of Salt Concentration. The pronounced effect of salts on enzyme I activity was shown to be largely due to (de)stabilization of the dimeric form of the enzyme. Although protein subunit–subunit interactions are expected to be sensitive to lyotropic affectors (Von Hippel & Schleich, 1969), it is still remarkable that such strong effects are produced by relatively low concentrations of salts. It is also remarkable that LiCl stimulates enzyme I activity and that it is as effective in doing so as KCl and NaCl, whereas these salts have different lyotropic effects on a great many enzymes, LiCl always being the stronger destabilizer [see Von Hippel & Schleich (1969) and references cited therein]. It seems likely therefore that some particular property of enzyme I causes the dimers to be stabilized by relatively low concentrations of salts, even by salts that are known destabilizers of native protein conformations. It could be that this unusual behavior of enzyme I is related to the unusual hydrophobic properties of this probably cytoplasmic enzyme (Robillard et al., 1979). Since the normal lyotropic effects of neutral salts seem to be a balance between salting in of polar groups and salting out of nonpolar groups (Robinson & Jencks, 1965; Nandi & Robinson, 1972a,b), a dominating role of hydrophobic interactions in the stability of dimeric enzyme I might account for the observed stabilization by neutral salts (LiCl, NaCl, and KCl all salt out nonpolar model compounds; Long & McDevit, 1952). Moreover, the observed relative effects of the tetraalkylammonium salts on enzyme I activity would be perfectly consistent with an important role of hydrophobic interactions in enzyme I dimerization. The hydrophobic properties of the cations increase with increasing alkyl chain length and so do their destabilizing effects on the active form of enzyme I.

Acknowledgments

We acknowledge the expert technical assistance of Ria ten Hoeve-Duurkens, who carried out all the phosphoryl-exchange experiments presented. We are grateful to Onno Misset for stimulating discussions and to Tom Nowak for critically reading the manuscript.

References

Brouwer, M., Elferink, M., & Robillard, G. T. (1982) *Biochemistry 21*, 82–88.

Hays, J. B. (1978) in *Bacterial Transport* (Rosen, B. P., Ed.) pp 43–102, Marcel Dekker, New York.

Hengstenberg, W. (1977) *Curr. Top. Microbiol. Immunol. 77*, 98–126.

Hoving, H., Lolkema, J. S., & Robillard, G. T. (1981) *Biochemistry 20*, 87–93.

Knowles, J. R. (1980) *Annu. Rev. Biochem. 49*, 877–919.

Kurganov, B. I. (1967) *Mol. Biol. (Moscow) 1*, 14–22.

Long, R. A., & McDevit, W. F. (1952) *Chem. Rev. 51*, 119–169.

Martell, A. E. (1964) *Spec. Publ.—Chem. Soc. No. 17*, 180–187, 634–640.

Mildvan, A. S., & Cohn, M. (1966) *J. Biol. Chem. 241*, 1178–1193.

Misset, O. (1981) Dissertation, University of Groningen, The Netherlands.

Misset, O., & Robillard, G. T. (1982) *Biochemistry* (following paper in this issue).

Misset, O., Brouwer, M., & Robillard, G. T. (1980) *Biochemistry 19*, 883–890.

Nandi, P. K., & Robinson, D. R. (1972a) *J. Am. Chem. Soc. 94*, 1299–1308.

Nandi, P. K., & Robinson, D. R. (1972b) *J. Am. Chem. Soc. 94*, 1308–1315.

Postma, P. W., & Roseman, S. (1976) *Biochim. Biophys. Acta 457*, 213–257.

Record, M. Th., Anderson, C. F., & Lohman, T. M. (1978) *Q. Rev. Biophys. 11*, 103–178.

Robillard, G. T., Dooyewaard, G., & Lolkema, J. S. (1979) *Biochemistry 18*, 2984–2989.

Robinson, D. R., & Jencks, W. P. (1965) *J. Am. Chem. Soc. 87*, 2470–2479.

Roseman, S. (1969) *J. Gen. Physiol. 54*, 138s–180s.

Saier, M. H. (1977) *Bacteriol. Rev. 41*, 856–871.

Saier, M. H., Schmidt, M. J., & Lin, Ph. (1980) *J. Biol. Chem. 255*, 8579–8584.

Stein, R., Schrecker, O., Lauppe, H. F., & Hengstenberg, W. (1974) *FEBS Lett. 42*, 98–100.

Valdes, R., & Ackers, G. K. (1979) *Methods Enzymol. 61H*, 125–142.

Von Hippel, P. H., & Schleich, Th. (1969) in *Structure and*

Stability of Biological Macromolecules (Timasheff, S. N., & Fasman, G. D., Eds.) pp 417–574, Marcel Dekker, New York.
Waygood, E. B., & Steeves, T. (1980) *Can. J. Biochem. 58*, 40–48.
Waygood, E. B., Meadow, N. D., & Roseman, S. (1979) *Anal. Biochem. 95*, 293–304.
Wold, F., & Ballou, C. E. (1957) *J. Biol. Chem. 227*, 301–312.
Zimmerman, J. K., & Ackers, G. K. (1971) *J. Biol. Chem. 246*, 7289–7292.

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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