Bartholmes concerning enzyme preparation as well as some supply of enzyme.

Registry No. Indole, 120-72-9; L-serine, 56-45-1; tryptophan synthase, 9014-52-2.

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Kinetics and Subunit Interaction of the Mannitol-Specific Enzyme II of the *Escherichia coli* Phosphoenolpyruvate-Dependent Phosphotransferase System†

F. F. Roossien, M. Blaauw, and G. T. Robillard*

ABSTRACT: Purified mannitol-specific enzyme II (EII^{mtl}), in the presence of the detergent Lubrol, catalyzes the phosphorylation of mannitol from P–HPr via a classical ping-pong mechanism involving the participation of a phosphorylated EII^{mtl} intermediate. This intermediate has been demonstrated by using radioactive phosphoenolpyruvate. Upon addition of mannitol, at least 80% of the enzyme-bound phosphoryl groups can be converted to mannitol 1-phosphate. The EII^{mtl} concentration dependence of the exchange reaction indicates that self-association is a prerequisite for catalytic activity. The self-association can be achieved by increasing the EII^{mtl} concentration or at low concentrations of EII^{mtl} by adding HPr or bovine serum albumin. The equilibrium is shifted toward the dissociated form by mannitol 1-phosphate, resulting in a mannitol 1-phosphate induced inhibition. Mannitol does not affect the association state of the enzyme. Both mannitol and mannitol 1-phosphate also act as classical substrate inhibitors. The apparent K_i of each compound, however, is approximately equal to its apparent K_m, suggesting that mannitol and mannitol 1-phosphate bind at the same site on EII^{mtl}. Due to strong inhibition provided by mannitol and mannitol 1-phosphate in the exchange reaction, the kinetics of this reaction cannot be used to determine whether the reaction proceeds via a ping-pong or an ordered reaction mechanism.

Mannitol-specific enzyme II (EII^{mtl})[1] from the *Escherichia coli* phosphoenolpyruvate-dependent phosphotransferase system catalyzes both the transport and concomitant phosphorylation of mannitol at the expense of PEP with the help of two intracellular proteins, HPr and EI (see Scheme I). This

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[1] Abbreviations: PEP, phosphoenolpyruvate; EII^{mtl}, mannitol-specific enzyme II; EII^{Glc}, glucose-specific enzyme II; DTT, dithiothreitol; PTS, phosphoenolpyruvate-dependent sugar phosphotransferase system; BSA, bovine serum albumin; mtl, mannitol; mtl-1-P, mannitol 1-phosphate; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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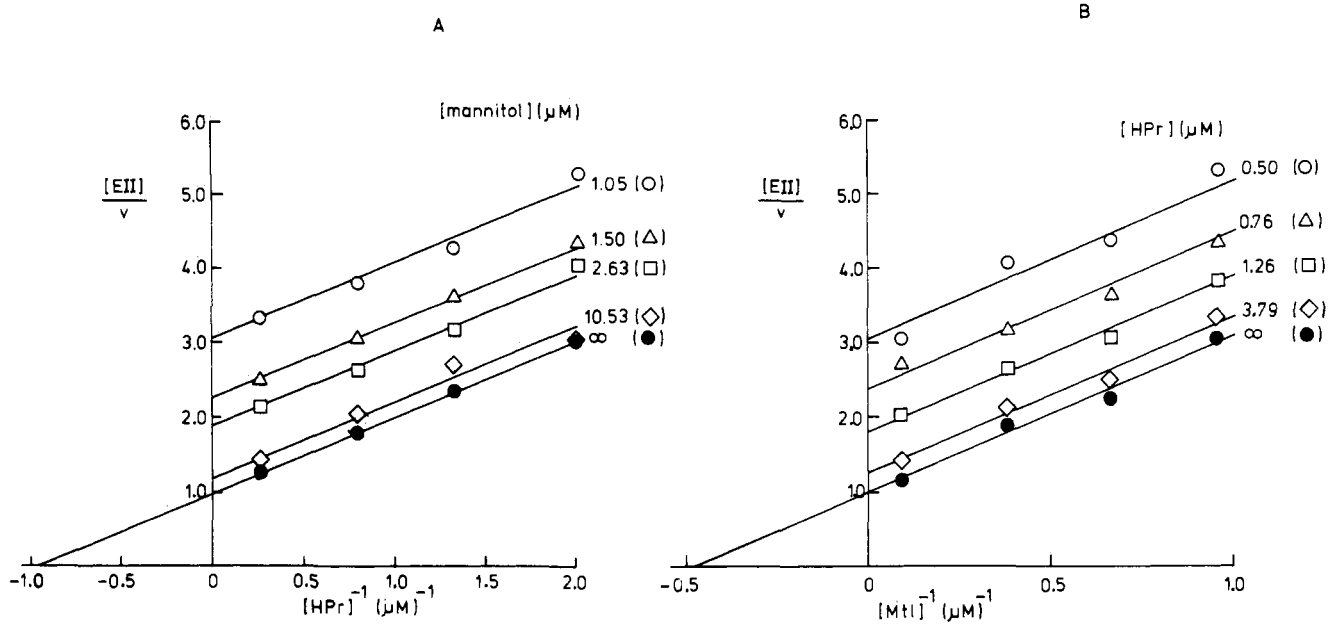

FIGURE 1: Lineweaver–Burk plots of the steady-state rates of mannitol phosphorylation as a function of the P–HPr (A) and mannitol (B) concentrations. The rates were determined by withdrawing aliquots of the reaction mixture at several times and measuring the amount of D-[1-^{14}C]mtl-1-P present as previously described (Misset et al., 1980). The reactions were carried out at 30 °C in mixtures containing 25 mM Tris-HCl buffer, pH 7.6, 1 mM DTT, 0.04% Lubrol, 5 mM $MgCl_2$, 10 mM NaF, 5 mM PEP, and 0.1 μM EI. The EII^{mtl} concentration was chosen such that not more than 10% of the sugar was converted during the course of the measurement. The values of $[EII]/V$ on the ordinate are expressed in units of (moles of EII minute per moles of mtl-1-P) × 10^3 where the EII concentration has been determined by the [^{14}C]pyruvate burst method (see Materials and Methods). The rates used to construct the curves for an infinite mannitol concentration in plot A (closed symbols) were obtained from the *y*-axis intercepts in plot B. Similarly, the data for an infinite P–HPr concentration in plot B were obtained from the *y*-axis intercepts in plot A.

EII differs from the glucose-specific EIIB^{Glc} in that it can apparently transfer the phosphoryl group directly from P–HPr to mannitol without the intervention of another protein factor, EIII. EII^{mtl}, purified to apparent homogeneity, is composed of a single polypeptide chain with a molecular weight in the range of 60000 (Jacobson et al., 1979). In the purified form,

Scheme I

$$PEP + HPr \xrightarrow{EI} P\text{–}HPr + Pyr \quad (1a)$$

$$P\text{–}HPr + mtl \xrightarrow{EII} HPr + mtl\text{-}1\text{-}P \quad (1b)$$

it is sufficient to catalyze the exchange process in Scheme II. Saier (1980) has proposed that this exchange reaction[2] is catalyzed by a dimeric form of EII^{mtl} because the rate of the reaction is linear with the square of the enzyme concentration. The phosphorylation reaction Scheme I, eq 1b), on the contrary, is linear with the first power of the enzyme concentration, which led Saier to propose that the phosphorylation reaction was catalyzed by the monomeric form of the enzyme. Since such a phenomenon poses interesting possibilities when considering active transport mechanisms, we have examined the kinetics of both the phosphorylation and exchange reactions catalyzed by the pure enzyme suspended in detergent micelles.

Scheme II

$$[^{14}C]mtl + mtl\text{-}1\text{-}P \xrightarrow{EII^{mtl}} mtl + [^{14}C]mtl\text{-}1\text{-}P \quad (2)$$

Materials and Methods

Bacteria. E. coli ML 308-225 were grown to OD_{550} 1–1.2 in medium 63 (Saier et al., 1976) containing 0.5% mannitol. Membrane fragments were prepared as described by Jacobson et al. (1979). EII^{mtl} was purified according to the method of Roossien & Robillard (1983). Enzyme I was purified according to Robillard et al. (1979) with the modifications described by Brouwer et al. (1982) and Misset & Robillard (1982). HPr was purified according to the method of Dooijewaard et al. (1979). The concentrations of EI, HPr, and EII were determined by using the [^{14}C]pyruvate burst procedure of Brouwer et al. (1981) (for details, see Results). The phosphorylation and exchange kinetics were measured at 30 °C (see figure legends for details). [^{14}C]Mannitol and [^{14}C]mannitol 1-phosphate were separated as described by Misset et al. (1980). The sodium salt of mtl-1-P was prepared by stirring a 2-mL suspension containing 100 mM mtl-1-P (barium salt) and 150 mM Na_2SO_4 for 2 h. The solid materials were removed by centrifugation, and the supernatant was loaded on a 2-mL column of Dowex AG1-X2 equilibrated in water. The column was washed with 30 mL of water, and the mtl-1-P was then eluted with 1 M ammonium bicarbonate. The mtl-1-P pool was diluted with 5 volumes of water and lyophilized. It was redissolved and relyophilized 2 times to remove the ammonium bicarbonate. The product was dissolved in H_2O and stored at −20 °C. The concentration of this mtl-1-P stock solution was determined by phosphate analysis according to the method of Chen et al. (1956). [^{32}P]Phosphoenolpyruvate was prepared by the method of Roossien et al. (1983).

Phosphoenolpyruvate monocyclohexylammonium salt and D-mannitol 1-phosphate barium salt were purchased from Sigma. D-[1-^{14}C]Mannitol (59 mCi/mmol) and [1-^{14}C]-phosphoenolpyruvate (12 mCi/mmol) were purchased from the Radiochemical Centre, Amersham.

Results

Phosphorylation Reaction. The dependence of the steady-state rate of phosphorylation has been measured as a function of the HPr and mannitol concentrations. In these

[2] Throughout this paper, we will use the following nomenclature for the different reactions: phosphoryl group transfer involves the overall, enzyme II catalyzed reaction PHPr + S ↔ S–P + HPr (Scheme I) whereas phosphoryl group exchange refers to the reaction in Scheme II.

measurements, the rates are linearly dependent on the EII^{mtl} concentration. EI and PEP are present in excess (see legend to Figure 1). The dependence of the rates on the mannitol and P–HPr concentrations is presented as Lineweaver–Burk plots in panels A and B of Figure 1. The patterns of parallel lines which are found are indicative of a ping-pong mechanism involving the participation of a phosphorylated intermediate, $P–EII^{mtl}$. The change in the rate as a function of the HPr concentration in Figure 1A is independent of the mannitol concentration. The converse is true for Figure 1B. These kinetics are consistent with the reaction sequence given below (Scheme III). The K_m for P–HPr extrapolated from Figure 1A at infinite mannitol concentration is 1 μM. The K_m for mannitol at infinite P–HPr concentration (Figure 1B) is 2 μM.

Scheme III

$$P–HPr + EII \rightleftharpoons EII·P–HPr \rightleftharpoons P–EII·HPr \rightleftharpoons P–EII + HPr \quad (3a)$$

$$P–EII + mtl \rightleftharpoons P–EII·mtl \rightleftharpoons EII·mtl\text{-}1\text{-}P \rightleftharpoons EII + mtl\text{-}1\text{-}P \quad (3b)$$

Demonstration of a Functional P–EII^{mtl} Intermediate. The evidence presented in the preceding section indicates that the EII-catalyzed phosphorylation proceeds via a ping-pong mechanism involving a functional $P–EII^{mtl}$ intermediate. Isolating such an intermediate and demonstrating that it can pass the phosphoryl group on to mannitol would be an even more convincing argument for its existence. Phosphorylated proteins of the PTS can be demonstrated by incubating these proteins with [^{14}C]PEP. When the protein becomes phosphorylated, a burst of [^{14}C]pyruvate is produced in an amount equivalent to the amount of phosphoenzyme formed. This method has been previously used to demonstrate the existence of P–HPr, P–EI, and $P–EIII^{Glc}$ (Hoving et al., 1981; Brouwer et al., 1982; Misset & Robillard, 1982; Scholte et al., 1981). A burst determination was carried out on EII^{mtl} in 25 mM sodium phosphate buffer, pH 7.0, containing 1 mM DTT, 10 mM NaF, 2.5 mM $MgSO_4$, 0.15 μM EI, 0.12 μM HPr, and 2 μM [^{14}C]PEP. After incubation for 5 min at 30 °C, EII^{mtl} was added which resulted in a burst in [^{14}C]pyruvate. A two-fold increase in the amount of EII^{mtl} added resulted in a 2-fold increase in the [^{14}C]pyruvate burst. These results confirm the formation of $P–EII^{mtl}$. Throughout the experiments described in this report, the [^{14}C]pyruvate burst method has been routinely used to determine the EII^{mtl} concentrations.

The functionality of $P–EII^{mtl}$ has been tested by its ability to transfer the phosphoryl group to mannitol. The method used is a pH drop procedure which we first developed to demonstrate a phosphorylated intermediate in a membrane preparation containing EII^{Glc} (Misset et al., 1983). This procedure is based on the observation that one of the reactions leading up to $P–EII^{Glc}$ is inhibited at pH 5 while the dephosphorylation of $P–EII^{Glc}$ by glucose still occurs at this pH. The same pH dependence has been reported for EII^{mtl} (Saier et al., 1977). In the experiment shown in Figure 2, the phosphorylated intermediates of HPr, EI, and EII^{mtl} are formed during a preincubation period. After preincubation, the pH was lowered to 5, and, at different time intervals, [^{14}C]mannitol was added, and the amount of [^{14}C]mannitol 1-phosphate produced was measured as a function of time. The experiment has been performed at two different EII^{mtl} concentrations. It clearly shows a mtl-1-P burst followed by a slow phosphorylation of mannitol. The latter indicates that the phosphorylation activity at pH 5 is not completely inhibited, as already shown by Jacobson et al. (1983). The mtl-1-P burst originates from contributions of both P–EI, P–HPr, and $P–EII^{mtl}$. As the EI and HPr concentrations of all samples are equal, the increment

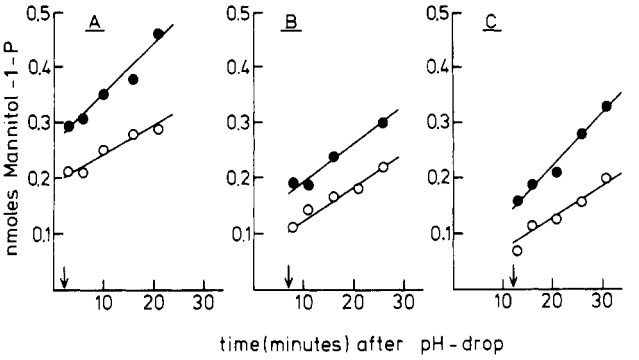

FIGURE 2: Mannitol 1-phosphate burst. The amount of mtl-1-P present in the reaction mixtures is plotted as a function of time after the pH drop (see text). Reaction mixtures (final volume 0.2 mL) contained 25 mM sodium phosphate buffer, pH 7.0, 1.0 mM DTT, 2.5 mM $MgCl_2$, 7.5 mM NaF, 20 μM PEP, 0.7 μM HPr, 0.15 μM EI, and 0.54 μM EII^{mtl} (open symbols) or 1.08 μM EII^{mtl} (closed symbols). After a 3-min 37 °C preincubation, at $t = 0$ the pH of the reaction mixtures was lowered to 5 by adding concentrated sodium acetate buffer, pH 4.5, to a final concentration of 62.5 mM. At $t = 2$ (A), 7 (B), or 12 min (C), [^{14}C]mannitol (final concentration 100 μM) was added to separate reaction mixtures, as indicated by arrows. At the indicated time intervals, samples were taken, and the amount of [^{14}C]mtl-1-P was determined. The mtl-1-P bursts are defined as the amount of [^{14}C]mtl-1-P formed at the moment [^{14}C]mannitol was added and are obtained by extrapolation of the above-shown time curves to respectively $t = 2$ (A), 7 (B), or 12 min (C).

in the burst between the lower and higher EII^{mtl} concentrations is a true measure of the amount of $P–EII^{mtl}$ present at the moment the sugar was added. If the logarithm of the difference in burst of the two EII^{mtl} concentrations is plotted against the time after the pH drop when [^{14}C]mannitol was added, a straight line is obtained (not shown). Extrapolation to $t = 0$ yields 0.72 mol of mtl-1-P formed per mol of $P–EII^{mtl}$.

Another way of demonstrating the existence of the $P–EII^{mtl}$ intermediate is shown in Figure 3. The Sephadex G75 elution profile of a reference sample in which EI and HPr were incubated in the presence of [^{32}P]PEP clearly shows the radioactivity peaks of ^{32}P-labeled P–EI (fractions 11–16) and P–HPr (fractions 21–26) (○). The amount of radioactivity in the P–EI and P–HPr peaks agrees very well with the amount of EI and HPr originally included in the reference sample. If in an identical experiment EII^{mtl} is included in the sample, the elution profile shows an increase in the radioactivity in the void volume of the column (△). This increase in radioactivity which coincides with the EII^{mtl} enzymatic activity peak clearly originates from the phosphorylated EII^{mtl} intermediate. In the experiment described in Figure 3, the incubation mix, which originally contained 35 pmol of EII^{mtl}, yielded, after chromatography, 29 pmol of ^{32}P-EII. Under these elution conditions (4 °C, pH 10), $P–EII^{mtl}$ appears to be rather stable to hydrolysis.

Other evidence for the existence of a phosphorylated EII^{mtl} intermediate comes from an experiment in which a sample containing the ^{32}P-labeled intermediates P–EI, P–HPr, and P–EII was electrophoresed in the presence of SDS (data not shown). The autoradiogram of the gel consisted of three well-resolved bands corresponding to P–EI, P–HPr, and $P–EII^{mtl}$.

EII^{mtl}-Catalyzed Exchange Reaction. The EII^{mtl} concentration dependence of the exchange reaction (Scheme II) is presented in Figure 4A (○). It is clear that the activity is not linear with enzyme concentration in the range of 0–10 pmol of EII^{mtl} per 100 μL of reaction mixture. On the other hand, the EII concentration dependence of the phosphorylation reaction (Scheme I) shown in the insert of Figure 4A is linear

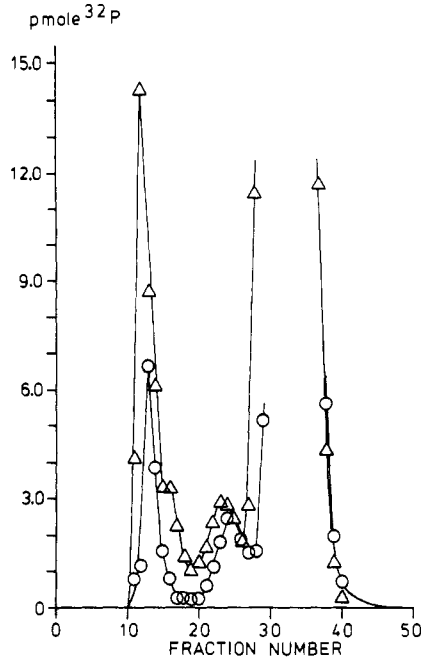

FIGURE 3: Isolation of [^{32}P]P–EII^{mtl}. Elution profile of ^{32}P-labeled P–EI and P–HPr (○). An 80-μL sample containing 0.17 μM EI, 0.24 μM HPr, 0.5 mM $MgCl_2$, 2.0 mM DTT, 50 mM sodium phosphate buffer, pH 7.0, and 8.4 μM [^{32}P]PEP was incubated for 10 min at 30 °C. Next it was eluted with 200 μL of cold elution buffer [20 mM Tris-HCl, pH 8.4, 1 mM DTT, 2 mM EDTA, and 0.1% Lubrol PX (v/v)] and immediately chromatographed on a Sephadex G75 column (39 × 1.1 cm), equilibrated in elution buffer (elution speed, 20 mL/h; fraction volume, 1 mL). Chromatography was perfomed at 4 °C. Fractions 11–16 contain [^{32}P]P–EI; fractions 21–28 contain [^{32}P]P–HPr. (△) Elution profile of a sample containing ^{32}P-labeled P–EI, P–HPr, and P–EII. The experiment was performed exactly as the previous one, except that 0.44 μM EII^{mtl} and 0.05% Lubrol were included in the incubated sample and the elution buffer consisted of 25 mM glycine–NaOH, pH 10.0, 1 mM DTT, 5 mM EDTA, and 0.1% Lubrol PX (v/v). The EII^{mtl} enzymatic activity peak, assayed with the PEP-dependent mannitol phosphorylation assay, coincides with the radioactivity peak in fractions 11–16.

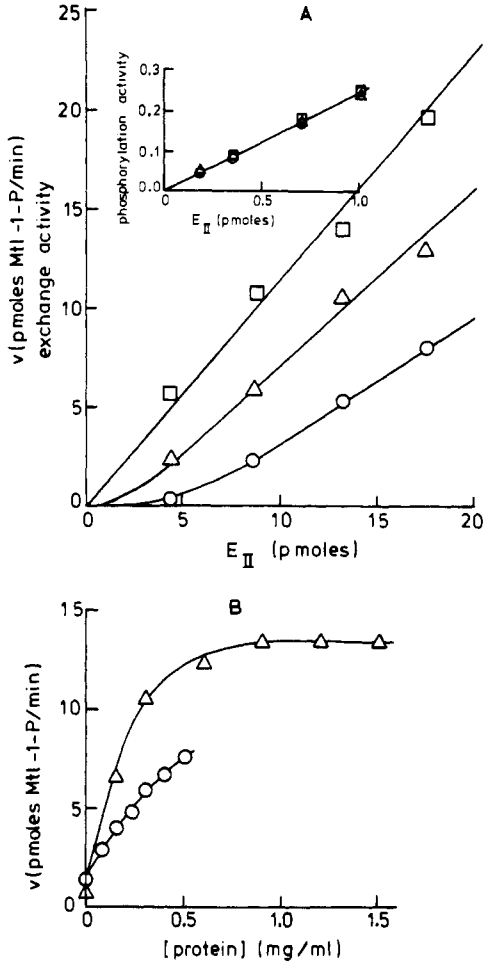

FIGURE 4: (A) EII^{mtl} concentration dependence of the exchange rate or (insert) the phosphorylation rate in the presence of HPr and/or BSA. The exchange activity was measured at 30 °C in 100-μL mixtures with the indicated amounts of EII^{mtl} in 25 mM sodium phosphate buffer, pH 6.2, containing 0.04% Lubrol PX, 1 mM DTT, 5 mM $MgCl_2$, 10 mM NaF, 0.6 mM mtl-1-P, and 4.0 μM [^{14}C]-mannitol: (○) no extra additions; (△) plus 0.3 mg of HPr/mL; (□) plus 1 mg of BSA/mL. The phosphorylation activity (insert) was measured at 30 °C in 100-μL mixtures with the indicated amounts of EII^{mtl} in 25 mM Tris-HCl, pH 7.6, containing 0.04% Lubrol PX, 1 mM DTT, 5 mM $MgCl_2$, 10 mM NaF, 5 mM PEP, 0.1 μM EI, 1.2 μM HPr, and 100 μM [^{14}C]mannitol: (○) no extra additions; (△) 0.1 mg of BSA/mL; (□) 1.0 mg of BSA/mL. (B) Influence of HPr (○) and BSA (△) on the exchange activity, at 30 °C, of 9 pmol of EII^{mtl} in 100 μL of 25 mM sodium phosphate, pH 6.2, containing 0.04% Lubrol, 1 mM DTT, 5 mM $MgCl_2$, 10 mM NaF, 1.2 mM mtl-1-P, and 4.0 μM [^{14}C]mannitol. The rates were derived from plots of the [^{14}C]mtl-1-P concentration found in the reaction mixture as a function of the incubation time.

even at enzyme concentrations in the range of 0.2–1.0 pmol of EII^{mtl} per 100 μL of reaction mixture. This difference in the EII^{mtl} concentration dependence of the exchange and phosphorylation reactions has already been reported by other authors (Saier, 1980; Leonard & Saier, 1983), who suggested that some self-association process (dimerization or oligomerization) was necessary for the exchange activity but not for the phosphorylation activity. The phosphorylation and exchange activities have been measured at their pH optima, pH 7.6 and 6.2, respectively. The choice of pH, however, is not the cause of the different enzyme concentration dependencies. The exchange reaction is not linear at both pHs. One reason for the observed differences could be the composition of the reaction mixtures. The phosphorylation kinetics are measured in the presence of excess EI, HPr, and PEP so that saturating quantities of P–HPr are continuously available. These components are not necessary and, therefore, not present during an exchange reaction. Figure 4B shows what happens to the rate of the exchange reaction when HPr (○) is added to the reaction mixture. HPr clearly increases the rate of the exchange reaction. In order to determine whether this effect was specific for a component of the PTS or a general protein concentration effect, we carried out a similar measurement using bovine serum albumin (△) instead of HPr. The stimulation in the rate is higher with BSA than with HPr. The observed stimulation could be due to an alteration of the specific activity of the unassociated form of EII^{mtl} or to an alteration in the equilibrium between associated and dissociated forms of EII^{mtl}, or to a combination of both effects. For this reason, the EII^{mtl} concentration dependencies of the exchange rates were measured in the presence of HPr and BSA (see Figure 4A). Concentrations of BSA which were saturating in Figure 4B altered the EII^{mtl} concentration dependence from nonlinear to linear and also altered the slope relative to the linear portion of the curve measured in the absence of BSA. These data suggest that both the equilibrium between the associated and unassociated forms of the enzyme and the specific activity of the associated form are affected by BSA. A nonsaturating HPr concentration also alters the EII^{mtl} concentration dependence. At 0.3 mg/mL HPr, there is still some nonlinearity, but the rates became more linear when the HPr/EII^{mtl} ratio was increased (not shown). The specific activity also appears to increase to the same level as found with

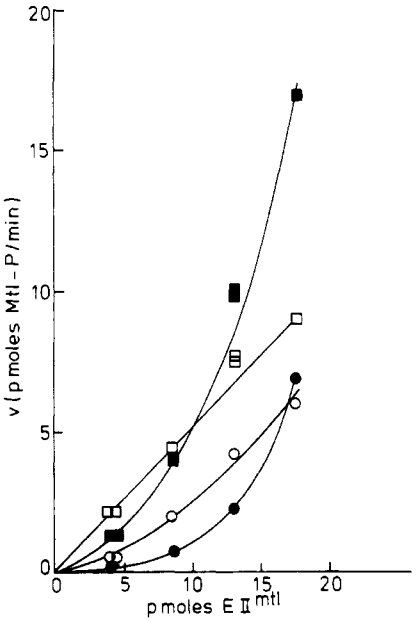

FIGURE 5: Influence of HPr and mtl-1-P on the EII^{mtl} concentration dependence of the exchange activity. The reactions were carried out at 30 °C in 100 μL of 25 mM sodium phosphate buffer, pH 6.2, containing 0.04–0.14% Lubrol PX, 1 mM DTT, 5 mM $MgCl_2$, 10 mM NaF, and 4.0 μM [^{14}C]mannitol. Open symbols: 0.3 mM mtl-1-P without HPr (○) or in the presence of 36 μM HPr (□). Closed symbols: 2.4 mM mtl-1-P without HPr (●) or in the presence of 36 μM HPr (■). The rates were determined as described in the legend of Figure 3.

BSA. No stimulation in the EII^{mtl} phosphorylation activity is observed when intermediate or saturating levels of BSA are used (insert of Figure 4A).

Influence of Mannitol and Mannitol 1-Phosphate on the Kinetic Properties of EII^{mtl}. (*A*) *Mannitol 1-Phosphate Concentration Dependence.* Mannitol 1-phosphate is the phosphoryl group donating substrate for the exchange reaction. At the same time, it is an inhibitor (Jacobson et al., 1983).

Inhibition could arise in at least two ways, the first being classical substrate inhibition in which mtl-1-P binds and interferes with the binding of mannitol to P–EII and the second being inhibition by shifting EII^{mtl} toward the dissociated state. In the previous section, we discussed the EII^{mtl} concentration dependence of the exchange reaction at a single mtl-1-P concentration, 0.6 mM in Figure 4A, and we showed that HPr or BSA was capable of changing the EII^{mtl} concentration dependence from nonlinear to linear. Figure 5 shows the influence of the mtl-1-P concentration on the EII^{mtl} concentration dependence. At low [mtl-1-P] in the absence of HPr (○), the dependence is nonlinear, but upon addition of 36 μM HPr (□), the rates become linear with EII^{mtl} concentration. At high [mtl-1-P] in the absence of HPr (●), the nonlinearity is even more striking so that the rates are even lower than at low [mtl-1-P]. Addition of 36 μM HPr (■) is not capable of removing the nonlinearity completely, so that, again, at low [EII^{mtl}], the rates are lower than found with low [mtl-1-P] in the presence of HPr. Similar data were found when BSA was used in place of HPr (data not shown). From these data, we conclude that one mode of inhibition exercised by mtl-1-P involves shifting the enzyme toward the dissociated state. Mannitol 1-phosphate favors the dissociated state; HPr and BSA favor the associated state. Whether or not mannitol 1-phosphate also inhibits by binding to EII^{mtl} (or P–EII^{mtl}) and preventing the binding of mannitol has not been determined because no experimental conditions have been found where an increase in the mtl-1-P concentration does not affect the association state of the enzyme at the same time.

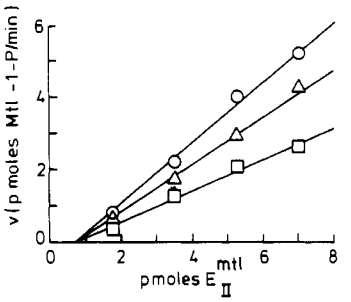

FIGURE 6: Influence of the mannitol concentration on the EII^{mtl} concentration dependence of the exchange rate. The exchange rates were measured at 30 °C in a 100-μL volume of 25 mM sodium phosphate buffer, pH 6.2, containing 0.04% Lubrol PX, 1 mM DTT, 5 mM $MgCl_2$, 10 mM NaF, 36 μM HPr, 0.6 mM mtl-1-P, the indicated amounts of EII^{mtl}, and 2 (○), 4 (△), or 8 (□) μM mannitol. The rates were determined as described in the legend of Figure 4.

(*B*) *Mannitol Concentration Dependence.* Mannitol is the phosphoryl group accepting substrate in the exchange reaction. Nevertheless, it is also a strong inhibitor of the exchange reaction with the purified enzyme (Jacobson et al., 1983).

Just as in the previous section, there are at least two modes of inhibition possible: (i) standard substrate inhibition in which mannitol binds to EII^{mtl} and prevents the binding of mtl-1-P and (ii) inhibition by affecting the association equilibrium of EII^{mtl}. As shown in Figures 4A and 5 when the association state is altered, an extrapolation of the linear portion of the curve to the abscissa shows that the point of intersection on the abscissa changes. This intercept is an indication of the EII concentration required to reach the associated, active state. The clearest evidence that mannitol does not alter the association state of EII^{mtl} is presented in Figure 6, where we have monitored the effect of changes in the mannitol concentration on the intercept. The reaction conditions are identical with those in Figure 4A (△). In the presence of 0.6 mM mtl-1-P, 36 μM HPr, and 4 μM mannitol, a small amount of nonlinearity is still apparent, indicating that EII^{mtl} is not fully associated at low concentrations. Changing the mannitol concentration in Figure 6 to 8 (□) or 2 (○) μM alters the slope of the curves but does not alter the intercept on the abscissa. These data suggest that mannitol inhibits by classical substrate inhibition rather than by influencing the association state of EII, as shall be discussed in the next section.

Discussion

The data presented in this report prove that the *E. coli* mannitol-specific enzyme II catalyzes the phosphorylation of mannitol via a ping-pong reaction during which the enzyme itself becomes phosphorylated at the expense of P–HPr. The [^{14}C]pyruvate burst and mtl-1-P burst, as well as the isolation of ^{32}P-labeled enzyme II by gel filtration, offer physical evidence for the occurrence of a catalytically effective P–enzyme II species. The K_m values for P–HPr and mannitol, defined at infinite concentrations of mannitol and HPr, respectively, are K_m(P–HPr) = 1 μM and K_m(mannitol) = 2 μM. Jacobson et al. (1983) recently reported a K_m for mannitol of approximately 11 μM.

The nonlinear dependence of the exchange rate on the EII^{mtl} concentration indicates that the enzyme can exist in two possible states: a dissociated form with low exchange activity and an associated form with relatively high exchange activity (Saier, 1980; Leonard & Saier, 1983). The dissociation–association equilibrium of EII^{mtl} is shifted to the associated form by the addition of HPr or BSA. In a preliminary publication, Leonard & Saier (1982) also report a stimulating effect of

BSA on the exchange rate. The fact that both the PTS protein HPr and BSA have the same kind of effect on the association of EII^{mtl} may mean that this association simply depends on the total protein concentration and is not specific for a given PTS protein such as HPr. On the other hand, P–HPr is the natural phosphoryl group donor for EII^{mtl}, and it is not unreasonable to suggest that it might play a specific role in the EII^{mtl} association process. In that case, we would expect it to be even more specific than HPr. Since mtl-1-P is the phosphoryl group donor in the exchange reaction, we cannot test the effect of P–HPr on the association state of the enzyme during the exchange reaction. Mannitol 1-phosphate works in a direction opposite to BSA and HPr; it shifts the dissociation–association equilibrium of EII^{mtl} toward the dissociated form. The effects of HPr or BSA and mtl-1-P are cumulative: at low EII^{mtl} concentration where a relatively high mtl-1-P concentration forces the enzyme into the inactive dissociated form, the addition of BSA or HPr shifts EII^{mtl} back to the active associated state. At relatively high mtl-1-P concentration, BSA or HPr was unable to prevent, completely, the mtl-1-P-induced inhibition. This may mean that mtl-1-P, besides affecting the dissociation of EII^{mtl}, acts as a substrate inhibitor as well, probably by binding at the sugar binding site.

Increasing mannitol concentrations have a strong inhibitory effect on the rate of the exchange reaction. Under the experimenal conditions described in Figure 6, the apparent affinity constant, K_m, and the inhibition constant, K_i, for mannitol are of the same order of magnitude, 0.5 and 1.5 μM, respectively. In the experiment shown, EII^{mtl} is shifted to the associated state by the addition of HPr. Figure 6 shows that this HPr-induced association state of EII^{mtl} is not influenced by the addition of mannitol. We argued above that HPr or BSA induces EII^{mtl} to reach the same association state which EII^{mtl} can reach by itself at higher enzyme concentrations. Therefore, we conclude that in contrast to mtl-1-P, the inhibition by mannitol does not originate from an effect on the dissociation behavior of EII^{mtl}. Probably mannitol inhibits by binding to EII^{mtl} and preventing the binding of mtl-1-P.

Saier (1980) suggested that the enzyme II catalyzed exchange reaction proceeds via a sequential type of mechanism, in contrast to the EII^{Glc}- and EII^{mtl}-catalyzed PEP-dependent sugar phosphorylation which proceeds via a ping-pong mechanism (Misset et al, 1983; this report). Analysis of the kinetics of the exchange reaction should discriminate between these two possible mechanisms: Lineweaver–Burk plots of the exchange rate as a function of substrate concentration would result in a set of parallel lines in the case of a simple ping-pong mechanism and in a set of intersecting lines in the case of a sequential type of mechanism. However, since the EII^{mtl}-catalyzed exchange reaction is subject to both substrate and product inhibition with inhibition constants (K_i) of the same order of magnitude as the affinity constant (K_m), it can be shown that data plotted in a Lineweaver–Burk plot do not permit any conclusion about the actual mechanism of the reaction. Therefore, other physicochemical methods will be necessary to determine the mechanism of the exchange reaction.

Registry No. PTS, 56941-29-8; EII^{mtl}, 37278-09-4; mtl, 69-65-8; mtl-1-P, 15806-48-1.

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