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Steady State Kinetics of Mannitol Phosphorylation Catalyzed by Enzyme II^mtl of the *Escherichia coli* Phosphoenolpyruvate-dependent Phosphotransferase System*

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The kinetics of mannitol phosphorylation catalyzed by enzyme II^mtl of the bacterial P-enolpyruvate-dependent phosphotransferase system are described for three different physical conditions of the enzyme, (i) embedded in the membrane of inside-out (ISO) oriented vesicles, (ii) solubilized and assayed above the critical micellular concentration (cmc) of the detergent, and (iii) solubilized and assayed below the cmc of the detergent. The kinetic characteristics of enzyme II^mtl, after solubilization of cytoplasmic membranes or after purification from these membranes are comparable. The mannitol-dependent kinetics at saturating concentration of P-HPr were biphasic both for the solubilized enzyme assayed above the cmc and for the enzyme in ISO vesicles. In contrast, the mannitol-dependent kinetics was monophasic for the solubilized enzyme assayed below the cmc. In the latter case, the maximal rate was about twice as high as observed with the two other conditions. The contribution of the high affinity phase to the maximal rate is lower for enzyme II^mtl in ISO vesicles than for the solubilized enzyme. At limiting concentrations of P-HPr, the kinetics is not according to the expected "ping-pong" mechanism.

Enzyme II^mtl is one of the transport proteins of the bacterial P-enolpyruvate[1]-dependent phosphotransferase system. This system is responsible for the uptake of numerous hexoses and hexitols into both Gram-negative and Gram-positive bacteria. The sugars are accumulated inside the bacterial cell by coupling transport and phosphorylation; the sugar appears as the corresponding sugar phosphate in the cytoplasm. The phosphoryl group originates from the high-energy metabolite P-enolpyruvate and is transferred to the sugar through a number of proteins, the last one being the transport protein itself (1, 2).

The mannitol-specific transporter, enzyme II^mtl, receives the phosphoryl group from a small protein, termed HPr. Therefore, the physiologically relevant reaction catalyzed by

enzyme II^mtl can be adequately described by the following.

$$\text{Mannitol}_{out} + \text{P-HPr} \xrightarrow{\text{II}^{mtl}} \text{mannitol-P}_{in} + \text{HPr} \qquad (\text{Eq. 1})$$

With respect to the substrate mannitol$_{out}$, the enzyme catalyzes two reactions, (i) transport across the membrane and (ii) phosphorylation. The emphasis in kinetic studies of this enzyme has been on the phosphorylation reaction catalyzed by the enzyme solubilized in detergent, where the discrimination between "in" and "out" is obscure (3–6). The most straightforward interpretation of the phosphorylation reaction in a noncompartmentalized system would be that exactly the same reaction is catalyzed as the one denoted in Equation 1, *i.e.* phosphorylation measures transport as well. However, since one of the two reactions of mannitol is invisible in this experimental setup this interpretation is not necessarily true. In fact, several lines of evidence indicate that the mechanism underlying the phosphorylation reaction is much more complex and a model showing the relation between the phosphorylation activity and the transport activity of the enzyme has been proposed (7). In this model two pathways for mannitol phosphorylation may be recognized, (i) binding to the periplasmically oriented binding site followed by translocation and phosphorylation and (ii) direct binding to the cytoplasmically oriented binding site followed by phosphorylation. Here we describe the kinetics of mannitol phosphorylation catalyzed by enzyme II^mtl under a number of different physical conditions that, potentially, assay these different kinetic pathways.

The data set generated in this paper will be used in the accompanying paper (8) to test a hypothetical kinetic scheme for enzyme II^mtl.

EXPERIMENTAL PROCEDURES

Materials

D-[1-³H]Mannitol (706.7 GBq/mmol) was purchased from New England Nuclear Research Products. D-[1-¹⁴C]Mannitol (2.2 GBq/mmol) was from Amersham. Decylpolyethylene glycol-300 (decylPEG) was synthesized by B. Kwant in our laboratory. The *Escherichia coli* phosphotransferase enzymes, E$_I$ and Hpr, were purified as described (9, 10).

Methods

Growth Conditions—*E. coli* strain ML308–225 was grown at 37 °C in medium 63 (11) containing 0.5% mannitol as the carbon source. Cells were grown in 5-liter flasks, filled with 2 liters of medium, and aerated by continuous shaking. Cells were harvested at A_{650} of 1.0.

Membrane Vesicles—These were prepared essentially as described (12). The vesicles were washed once with 25 mM Tris, pH 7.6, 1 mM dithiothreitol, 1 mM NaN$_3$. Aliquots of 50 μl containing 1 mg/ml membrane protein (13) were stored in liquid nitrogen. Samples were thawed rapidly before use. Each sample was used only one time.

[1] The abbreviations used are: P-enolpyruvate, phosphoenolpyruvate; ISO inside-out; decylPEG, decylpolyethylene glycol 300; cmc, critical micellular concentration.

Enzyme II^mtl—This was purified from these membranes as described (4) with modifications (5).

Activity Measurements—All experiments were performed at 30 °C. The activity of enzyme II^mtl was measured by following the formation of [^3H]mannitol-P or [^14C]mannitol-P in time in a total volume of 100 μl. The buffer contained 25 mM Tris, pH 7.5, 5 mM dithiothreitol, and 5 mM MgCl₂. Four samples of 20 μl each were withdrawn at consecutive times and analyzed for labeled mannitol-P as described (5). A fifth sample of 10 μl was used to relate the labeled mannitol concentration to the total radioactivity in the sample. A 20-μl sample of 0.8 μM [^3H]mannitol or 100 μM [^14C]mannitol contained 134,000 or 94,100 cpm, respectively. The error in these numbers is typically 1–2%. The procedure results in a time-independent background of 0.26 and 0.38% for [^3H]mannitol and [^14C]mannitol, respectively. The amount of mannitol-P formed was usually less than 10% of the initial mannitol concentration to assure initial rate measurements. P-enol-pyruvate-dependent phosphorylation was initiated by addition of labeled mannitol to the enzyme suspension incubated with 5 mM P-enolpyruvate, 0.22 μM enzyme I, and the appropriate concentration of HPr. In the overall reaction the phosphoryl group donor for enzyme II^mtl is P-HPr, which is rephosphorylated by P-enolpyruvate in a reaction catalyzed by enzyme I. The concentrations of enzyme I and P-enolpyruvate were chosen such that essentially all HPr was in the phosphorylated state under all turnover conditions. Therefore, the concentration of the substrate P-HPr equaled the concentration of HPr added initially to the mixture.

Membranes were solubilized by suspending ISO membranes in a buffer containing 25 mM Tris, pH 7.5, 5 mM dithiothreitol, and 0.2% decylPEG at a membrane protein concentration that was 20 times higher than used in the activity measurement. Subsequently, these solubilized membranes were diluted 20 times into the assay mixture, with or without 0.25% decylPEG for assays above and below the cmc of the detergent, respectively.

Evaluation of the Data—Rates were estimated from best fits of a straight line through the four consecutive time points and the background. The kinetic data was subjected to nonlinear fit procedures and the residual errors were analyzed graphically (14, 15). A residual is defined as the difference between the experimental and the calculated value divided by the calculated value $\delta = (y_{exp} - y_{calc})/y_{calc}$. The mean residual error of n data points is defined by $\Sigma(ABS\ \delta_i)/n$. Values for kinetic parameters are averages from at least two independent experiments.

RESULTS

Kinetics of Mannitol Phosphorylation Catalyzed by Enzyme II^mtl Solubilized in DecylPEG—We routinely purify enzyme II^mtl in the presence of decylPEG, a detergent with a relatively low cmc of 0.035% (v/v). The enzyme is stable for prolonged periods of time in the presence of decylPEG concentrations above the cmc. Activity measurements indicate that the turnover rate of enzyme II^mtl is independent of the decylPEG concentration over a large range of concentrations above the cmc (not shown). A concentration of 0.25% decylPEG was chosen in the first series of experiments. The buffer contained 25 mM Tris, pH 7.5, 5 mM MgCl₂, and 5 mM dithiothreitol. In this buffer the enzyme is in the dimeric state over a wide range of turnover conditions (6).

The rate of mannitol phosphorylation by P-HPr catalyzed by cytoplasmic membranes solubilized with decylPEG was measured over a range of mannitol concentrations from 1.25 to 640 μM in two independent experiments. The P-HPr concentration was 24 μM which is close to saturating (see below). The data from the two experiments were separately fitted to a single saturation curve resulting in mean residual errors of 34 and 29%. Analysis of the residuals shows a similar nonrandom distribution for both sets of data, indicating that the experimental data is not well described by a single saturation curve (Fig. 1, *inset A*). A double-reciprocal plot of the data clearly shows biphasic kinetics (Fig. 1, *main plot*). In the concentration range of 1.5 μM to about 5 μM the data fit within experimental error to a single saturation curve (the
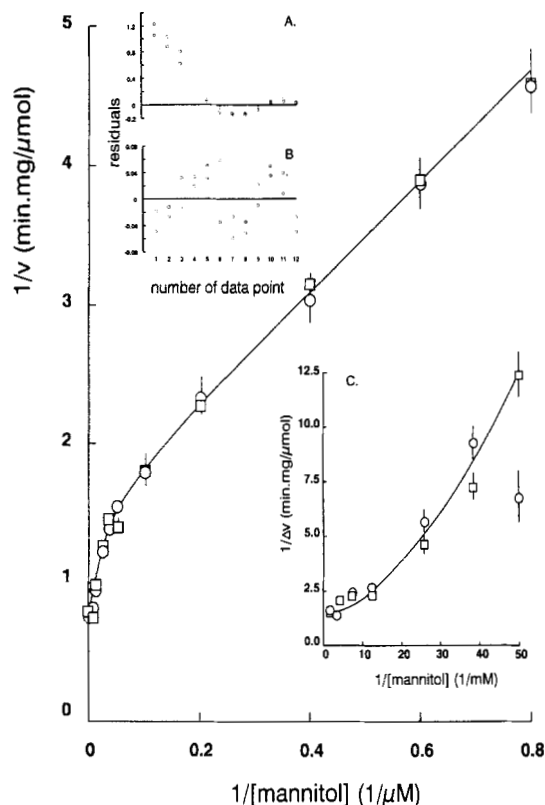

FIG. 1. **Kinetics of mannitol phosphorylation catalyzed by cytoplasmic membranes solubilized in decylPEG.** The rate of mannitol phosphorylation (*v*) was measured at mannitol concentrations of 1.25, 1.67, 2.5, 5, 10, 20, 26.7, 40, 80, 160, 320, and 640 μM. The P-HPr concentration was 24 μM. The membrane protein concentration and the interval between the time points were adjusted according to the expected rate, ranging from 83.8 to 250 ng/ml and 2.5 to 20 min, respectively. The *main plot* shows the data plotted according to Lineweaver-Burk (○ and □, duplicate experiments). *Inset A*, residual analysis after fitting the data to a single saturation curve. The data points are numbered as listed above. *Inset B*, residual analysis after fitting the data to the sum of two saturation curves. *Inset C*, analysis of the low affinity phase. The difference of the actual rate and the extrapolated rate from the high affinity regime (Δ*v*) was plotted in a Lineweaver-Burk plot. The markers in the insets (○ and □) correlate with those in the main plot.

mean residual error is 0.9%) resulting in an affinity constant for mannitol $K_M^{mtl} = 2.45$ μM and a maximal rate of 644 nmol/min·mg membrane protein. We will refer to mannitol concentrations below and above 5 μM as the high and low affinity regime, respectively. Subsequently, the data from the two experiments was fitted separately to the sum of two saturation curves resulting in mean residual errors of 3.4 and 3.2%. In spite of these small errors, analysis of the residuals (Fig. 1, *inset B*) shows that the residuals are not randomly distributed around zero. Instead, the residuals of the two experiments cluster similarly around the x axis suggesting that the low affinity regime is not described adequately by a saturation curve. *Inset C* in Fig. 1 shows the low affinity regime when analyzed as the difference between the measured rates and the rates calculated with the parameters from the high affinity regime. The low affinity regime manifests itself most strongly in the mannitol concentration range from 20 to 100 μM. Phenomenologically, the mannitol concentration giving half the maximal rate is about 60 μM.

The affinity for P-HPr at a saturating mannitol concentration of 1 mM was estimated from the rates measured between

FIG. 2. **Kinetics of mannitol phosphorylation catalyzed by solubilized membranes in the high affinity domain.** The rate of mannitol phosphorylation (v) was measured at concentrations of mannitol of 0.2, 0.27, 0.4, 0.5, 0.67, 0.8, 1.0, and 2.0 μM. The P-HPr concentrations were 3 (●), 0.6 (O), 0.3 (■), and 0.1 (□) μM. The membrane protein concentration in the experiments were either 25 or 50 ng/ml. The *left plot* shows the data plotted according to Lineweaver-Burk. The *right plots* show the residuals of the data at the four P-HPr concentrations after fitting the entire data set to the rate equation pertinent to a ping-pong mechanism. The data points are numbered as listed above. The *markers* in the plots on the right correlate with those in the *left plot*.
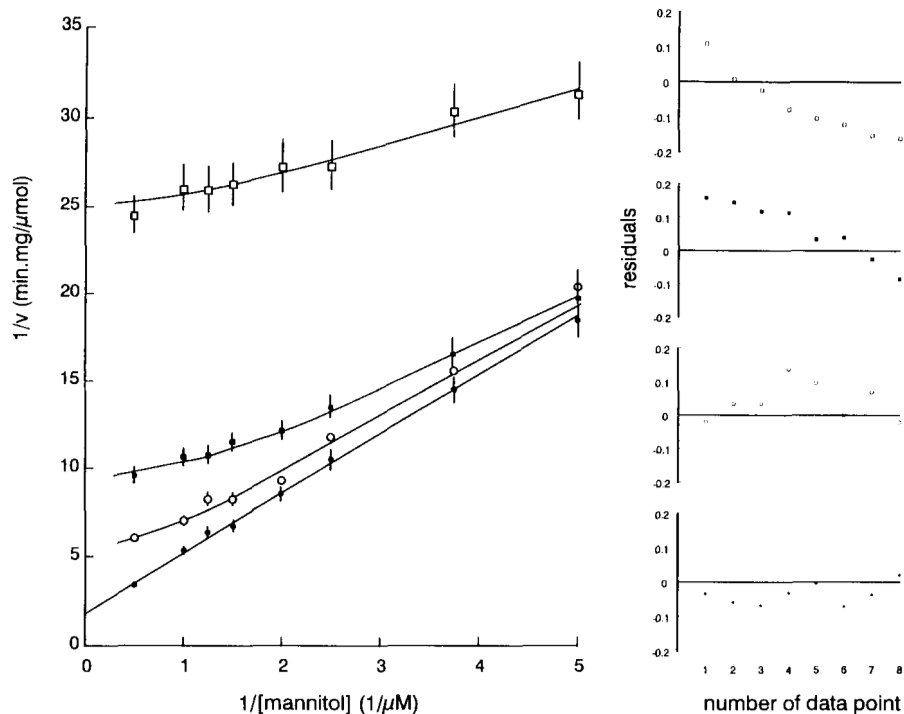
TABLE I

Kinetic parameters for P-enolpyruvate-dependent mannitol phosphorylation catalyzed by enzyme II^{mtl} under different physical conditions

The kinetic parameters of purified enzyme II^{mtl}, enzyme II^{mtl} solubilized from ISO vesicles, and assayed above the cmc of decylPEG (solubilized), enzyme II^{mtl} embedded in the membrane of ISO vesicles (ISO vesicles), and enzyme II^{mtl} solubilized from ISO membranes and assayed below the cmc of decylPEG are presented. The parameters are described in the text. The affinity constants are in μM, the maximal rates in min^{-1} and nmol/min·mg for the purified enzyme and the other conditions, respectively.

	Purified	Solubilized	ISO vesicles	Below the cmc
K_M^{mtl} (HA)	3.9 ± 0.8[a] (5)[c]	2.3 ± 0.3 (6)	9.5 ± 0.5 (4)	—[b]
V_{max} (HA)	700 ± 100 (5)	570 ± 74 (6)	214 ± 25 (4)	—[b]
K_M^{mtl} (LA)	—[d]	—[d]	—[d]	95 ± 12 (3)
$K_M^{P\text{-}HPr}$ (LA)	6.3 ± 0.6 (3)	4.7 ± 0.6 (3)	15 ± 3 (5)	10.6 ± 2.6 (2)
V_{max} (TOT)	2800 ± 300 (3)	1940 ± 200 (5)	1770 ± 120 (5)	3900 ± 600 (3)

[a] Standard deviation.
[b] Single low affinity phase.
[c] Number of experiments.
[d] Nonlinear Lineweaver-Burk plot.

3 and 24 μM P-HPr. The rate increased according to a single saturation curve with an affinity constant for P-HPr, $K_M^{P\text{-}HPr}$, of 4.7 ± 0.6 μM (Table I).

The results thus far suggest that the kinetic behavior of enzyme II^{mtl} may be described by four parameters. In the high affinity regime, the affinity constant for mannitol, K_M^{mtl}(HA), and the maximal rate, V_{max}(HA), and in the low affinity regime, the affinity constant for HPr, $K_M^{P\text{-}HPr}$(LA), and the overall maximal rate, V_{max}(TOT). The four parameters may be estimated from the saturation behavior of the rate between 1 and 5 μM mannitol at saturating concentrations of P-HPr (K_M^{mtl}(HA) and V_{max}(HA)) and between 3 and 24 μM P-HPr

at saturating concentrations of mannitol (Table I). These four parameters were determined again after purification of enzyme II^{mtl} from the membranes. The results were qualitatively the same as observed with the solubilized membranes (Table I). The presence of the low affinity regime causes the turnover number of the enzyme to be a factor of 2–3 higher than reported before (4).

In an earlier paper from our laboratory (4), the rate of mannitol phosphorylation catalyzed by purified enzyme II^{mtl} solubilized in the detergent Lubrol PX100 was measured in the mannitol concentration range from 1 to 10 μM, which in part extends into the low affinity regime described above. Different concentrations of P-HPr resulted in a set of parallel lines when analyzed in Lineweaver-Burk plots in accord with the expected "ping-pong" type of mechanism (4). We have repeated these experiments in the mannitol concentration range from 0.2 to 2 μM in which there is no interference from the low affinity regime, and with an extended range of P-HPr concentrations. The whole data set was fitted to the rate equation pertinent to the ping-pong mechanism resulting in a mean residual error of 7%. Graphical analysis of the residuals revealed a nonrandom deviation of the experimental data from the rate equation for a ping-pong mechanism (Fig. 2, *right*). A double-reciprocal plot of the data shows that with 3 μM P-HPr the data can adequately be described by a single saturation curve (●). This line parallels the line in the high affinity regime at 24 μM P-HPr shown in Fig. 1. However, lowering the P-HPr concentration below 3 μM seems to affect the rate at the higher mannitol concentrations relatively more than at the lower concentrations, resulting in nonlinear relationships. This abnormal behavior was confirmed by measuring the rate in the P-HPr concentration range from 0.25 to 24 μM at a fixed mannitol concentration of 5 μM (Fig. 3). Analysis of the residuals after fitting the data to a single saturation curve reveals a nonrandom distribution (*insert*, ●). The double-reciprocal plot shows that the data fit reasonably well to a single saturation curve between 1 and 24 μM (mean residual error of 2.5%). The affinity constant for P-HPr equals
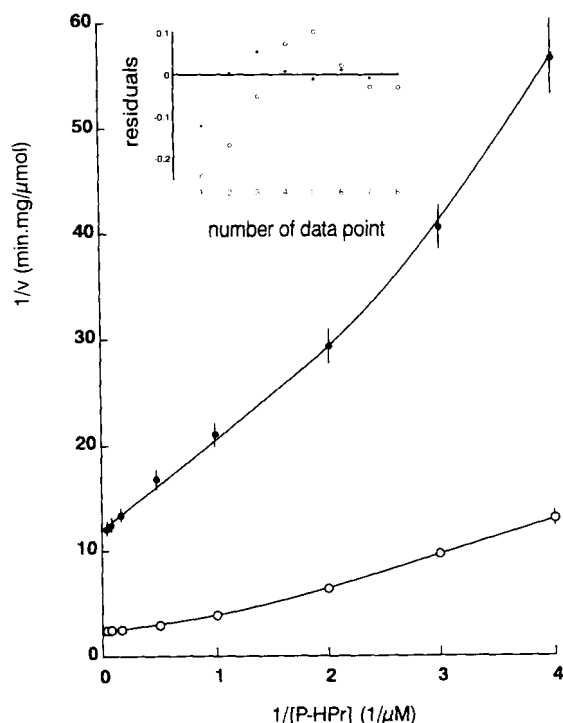
FIG. 3. **P-HPr-dependent kinetics of mannitol phosphorylation in the high affinity domain.** The rate of mannitol phosphorylation catalyzed by ISO membranes (●) and ISO membranes solubilized in 0.25% decylPEG (○) was measured at P-HPr concentrations of 0.25, 0.33, 0.5, 1, 2, 6, 12, and 24 μM. The mannitol concentration was 5 μM. *Main plot*, the data plotted according to Lineweaver-Burk. *Inset*, residual analysis after fitting the data to a single saturation curve. The data points are numbered as listed above. The *markers* correlate with those in the *main plot*.

0.59 μM. However, below 1 μM the rate decreases more rapidly than expected.

Kinetics of Mannitol Phosphorylation Catalyzed by Enzyme II^{mtl} Embedded in the Membrane of ISO Vesicles—In the high affinity regime, the rates of mannitol phosphorylation by enzyme II^{mtl} embedded in the membrane of ISO vesicles is much lower than observed with the solubilized enzyme (Fig. 4, ○ and ×, respectively). The data up to 5 μM mannitol fits to a single saturation curve with a mean residual error of 1.6%. The affinity constant for mannitol K_M^{mtl}(HA) equals 9.3 μM and the maximal rate was 234 nmol/min·mg. The low affinity regime shows a pronounced second phase, resulting in much smaller differences between the rates catalyzed by the ISO vesicles and the solubilized enzyme. Moreover, analysis of the saturation behavior of the rate between 3 and 24 μM P-HPr at a saturating mannitol concentration of 1 mM yielded an affinity constant for P-HPr of K_M^{mtl}(LA) = 15 ± 3 μM for the ISO vesicles *versus* 4.7 ± 0.6 μM for the solubilized enzyme. As a consequence, the maximal rate in the two conditions of the enzyme (V_{max}(TOT)) is about equal (Table I).

The affinity constant for mannitol in the high affinity regime is significantly different with the solubilized enzyme and the enzyme in ISO membranes, K_M^{mtl}(HA) = 2.3 ± 0.3 and 9.3 ± 0.5 μM, respectively (Table I). In contrast, the behavior with respect to P-HPr was quite similar for the two conditions of the enzyme (Fig. 3, ○ and ●) even though the deviation from a single saturation curve with the ISO membranes was less pronounced. In the concentration range of 1 to 24 μM P-HPr the data for the membrane bound enzyme (●) fitted a saturation curve with a mean residual error of 0.8% and an
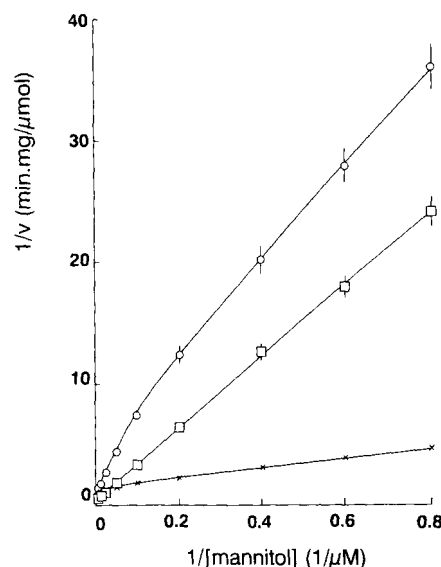

FIG. 4. **Comparison of the kinetics of mannitol phosphorylation catalyzed by enzyme II^{mtl} under different physical conditions.** ISO membrane vesicles were assayed in the absence of decylPEG (○), after solubilization in the presence of 0.25% decylPEG (×), and after solubilization in the presence of 0.01% decylPEG (□). The P-HPr concentration was 24 μM. The rates of the experiments with the ISO vesicles (○) and in 0.25% decylPEG (×) are averages of two independent experiments.

affinity constant of 0.84 μM compared to 0.59 μM for the solubilized membranes. Below this concentration range the rate fell more rapidly than expected. The most important difference between the kinetics seems to be that, over the range of P-HPr concentrations tested, the rates with the solubilized enzyme were a factor of 5 higher than observed with the membrane bound enzyme.

Kinetics of Mannitol Phosphorylation Catalyzed by Enzyme II^{mtl} Diluted Under the CMC of DecylPEG—Solubilization of cytoplasmic membranes with 0.25% decylPEG followed by dilution to 0.01% decylPEG which is below the cmc of the detergent, renders enzyme II^{mtl} in a meta-stable state. The rate of mannitol phosphorylation catalyzed by the enzyme under these conditions was stable for about 1 h after which the rate reduced gradually, probably because of precipitation of the enzyme. Addition of decylPEG during this first hour resulted in an enzyme with the same activity as observed before the dilution step indicating complete reversibility of the treatment.

In the range from 1.25 to 640 μM mannitol and at 24 μM P-HPr the kinetics of enzyme II^{mtl} under this physical condition is fitted by a single saturation curve with a residual error of 1.9% and an affinity constant for mannitol of 91 μM (Fig. 4, □). The maximal rate, inferred from a P-HPr titration at saturating mannitol concentration, is twice as high as observed with the enzyme solubilized and measured above the cmc or with the ISO vesicles (Table I). Lowering of the P-HPr concentrations results in decreasing fits to a single saturation curve (Fig. 5, *right*). Lowering the P-HPr concentration from 12 (●) to 0.5 μM (□) did not have a significant effect on the rate in the range from 2 to 5 μM mannitol but it lowered the rate dramatically at the higher mannitol concentrations. At the lower mannitol concentration limit, the kinetics followed a similar saturation curve but as the P-HPr concentration was lowered, it seemed to set a limit to the maximal rate at infinitely high mannitol concentrations.

DISCUSSION

The main kinetic characteristics of enzyme II^{mtl} described in this paper are, (i) the biphasic mannitol-dependent kinetics

FIG. 5. **Kinetics of mannitol phosphorylation catalyzed by solubilized membranes under the cmc of the detergent.** Cytoplasmic membranes were solubilized in 0.25% decylPEG and subsequently diluted to 0.01% decylPEG in the assay mixture. Final membrane protein concentration: 250 ng/ml. The rate of mannitol phosphorylation was measured at mannitol concentrations of 2, 2.67, 4, 8, 16, 32, 64, and 128 μM. The P-HPr concentrations were 0.5 ($\square$), 1 ($\blacksquare$), 3 (O), and 12 ($\bullet$) μM. *Left plot*, the data plotted according to Lineweaver-Burk. *Right plots*, residual analysis after fitting the data at each P-HPr concentration separately to a single saturation curve. The data points are numbered as listed above. The *markers* correlate with those in the *main plot*.
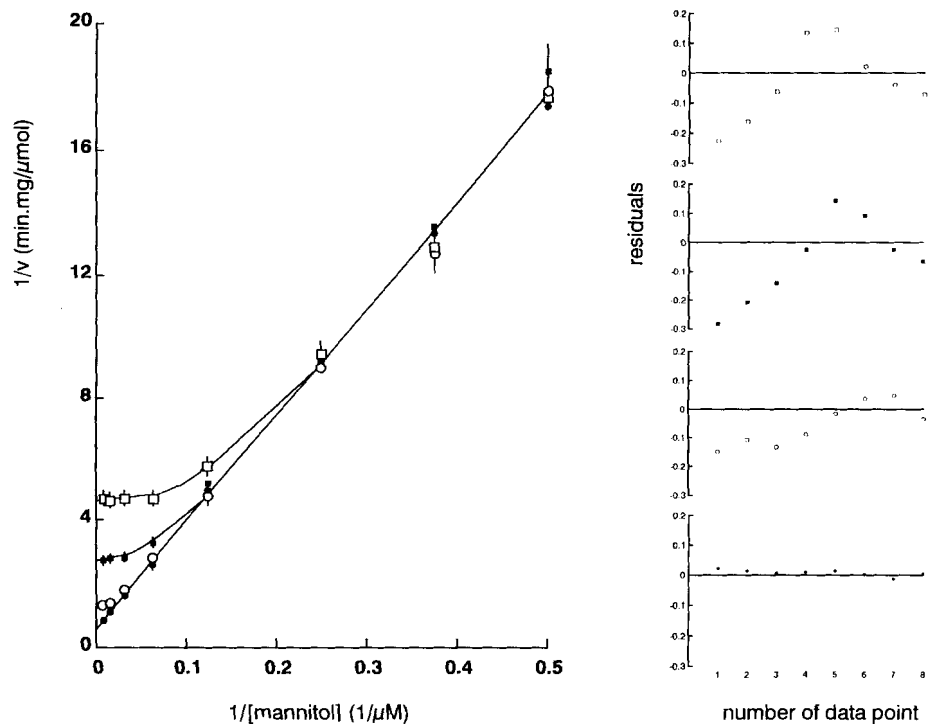
of both the solubilized enzyme and the ISO vesicles, (ii) the stimulation of the rate in the high affinity regime following solubilization of ISO vesicles, (iii) the similar maximal rates for solubilized enzyme and ISO vesicles, (iv) the deviation from ping-pong kinetics at limiting concentrations of P-HPr, (v) the monophasic kinetics of the solubilized enzyme diluted under the cmc at saturating P-HPr, (vi) the deviation from saturation kinetics at P-HPr limitation under the same condition, and (vii) the 2-fold increase of the maximal rate under this condition. In the accompanying paper (8) a hypothetical kinetic model for enzyme II^{mtl} will be presented and the present data will be used to test the kinetic performance of the model. In this "Discussion" section only experimental aspects of the results and the interpretation of the different physical conditions will be discussed.

Cytoplasmic Membranes as a Source of Enzyme II^{mtl}—We have determined the kinetics of mannitol phosphorylation catalyzed by enzyme II^{mtl} using cytoplasmic membranes derived from *E. coli* cells induced for the mannitol transport system as the source of the enzyme. This has the advantage that the kinetic behavior of the enzyme can be compared directly under different physical conditions. Nevertheless, care should be taken that enzyme II^{mtl} is solely responsible for the kinetics. Therefore, a number of the experiments carried out on solubilized membranes were repeated with purified enzyme II^{mtl} in the detergent decylPEG. Table I shows that the biphasicity of the mannitol-dependent kinetics pertinent to the solubilized cytoplasmic membranes is retained with the purified enzyme, indicating that it is a property of enzyme II^{mtl} and not caused by some other component present in the membrane preparation. Other experiments with the purified enzyme showed that all the reported properties could be ascribed to enzyme II^{mtl} (not shown). Apparently, enzyme II^{mtl} behaves like an independent entity after solubilization from cytoplasmic membranes, a physical condition comparable with the purified enzyme kept soluble in detergent.

Homogeneity of the Enzyme Population—Enzyme II^{mtl} is

the only mannitol-specific transport protein known to be expressed during growth of *E. coli* on mannitol. Nevertheless, inhomogeneity in the enzyme II^{mtl} population may arise during the isolation procedures of the membranes or the enzyme. Biphasic kinetics might be indicative for such inhomogeneity since the resulting kinetics would be a summation of the kinetics of the different populations of enzyme molecules. Residual analysis suggests that the mannitol-dependent kinetics (Fig. 1) is a property of a single population rather than a summation of two independent populations. Moreover, the contribution of the high affinity phase to the overall maximal rate ($V_{max}(HA)/V_{max}(TOT)$, Table I) is not significantly different for solubilized ISO membranes and for enzyme II^{mtl} purified from these membranes. No shift between two hypothetical populations can be detected during the purification procedure which is more in line with a homogeneous enzyme population.

Different Physical Conditions for the Enzyme—The kinetic pathways of an enzyme may be better understood by monitoring the kinetic properties under different physical conditions. In the case of a transport protein where a substrate molecule is transported from one compartment to another, the presence or absence of substrate in either one or both of the two compartments is likely to have its effect on the kinetic behavior. Although the two compartments have disappeared after solubilization of cytoplasmic membranes, enzyme II^{mtl} will still have its "cytoplasmic" and "periplasmic" side. However, both sides of the protein see exactly the same concentration of mannitol. On the other hand, with enzyme II^{mtl} embedded in the membrane of ISO vesicles the two sides of the protein face two different compartments, the internal and external volume. The external concentration of mannitol can be controlled experimentally. The internal mannitol concentration is determined by the activity of the enzyme, assuming that passive diffusion of mannitol over the membrane is negligible (16, 17) and is likely to be different from the external concentration.

The results indicate a remarkable difference between the

kinetics of solubilized enzyme II^{mtl} when determined above or below the cmc of the detergent. Since, under both conditions, there is no compartmentalization, the different kinetics must reflect differences within the enzyme itself, caused by the different surrounding of the hydrophobic part of the enzyme. The microenvironment of the hydrophobic membrane domain provided by the detergent micelle or the phospholipid bilayer may be comparable, allowing the same mobility in terms of conformational changes. Breakdown of the micelle surrounding the enzyme by lowering the detergent concentration below the cmc may reduce this mobility to such an extent that transitions of the hydrophobic domain are inhibited. More specifically, conformational changes involved in the translocation of mannitol may be blocked. This tentative interpretation is supported by the notion that the kinetics at saturating concentration of P-HPr shows two phases above the cmc and one below the cmc (Fig. 4, $\times$ and $\square$, respectively) suggesting that part of the kinetic scheme is inaccessible below the cmc.

Homogeneity of the ISO Vesicle Preparation—Membrane vesicles prepared by passing cells through a French press have predominantly an inside-out orientation, but a small fraction of vesicles with a right-side-out orientation and open structures will be present (18, 19). Enzyme II^{mtl} molecules in the right-side-out vesicle fraction will not contribute to the rate of phosphorylation of mannitol since the internal volume is not accessible to P-enolpyruvate, enzyme I, and HPr. The physical condition of enzyme II^{mtl} in membranes that do not form closed vesicles is identical to the solubilized enzyme with respect to the substrate mannitol (see above). Therefore, the kinetics of mannitol phosphorylation catalyzed by the ISO membrane preparation is likely to be a summation of the kinetics of ISO vesicles and the kinetics of open membrane sheets that will be similar to the kinetics of the solubilized enzyme (20). The high affinity regime in the mannitol-dependent kinetics catalyzed by the ISO membrane preparation could arise from the open membrane structures (Fig. 4, $\bigcirc$ and $\times$). Then, the real kinetics of the ISO vesicles would be described by the low affinity regime. In the accompanying paper (8) it will be demonstrated that this interpretation is consistent with the proposed kinetic model.

Subtraction of the high affinity regime from the overall kinetics of the ISO membrane preparation would leave the kinetics of the ISO vesicles, but, unfortunately, it also contains the low affinity contribution of the open membrane

structures. The different affinity constants for mannitol in the high affinity regime observed with the solubilized membranes and the open structures in the ISO membrane preparation (K_M^{mtl}(HA) = 2.4 and 9.3 μM, respectively) may reflect the slightly different microenvironment of enzyme II^{mtl} under the two conditions. The drawback of this is that the contribution of the open structures to the low affinity regime of the kinetics of the ISO membrane preparation cannot be quantified in any reliable way. It can only be concluded that the affinity for mannitol is relatively low at saturating concentrations of P-HPr and that the maximal rate is similar to that observed with the solubilized membranes.

The similar P-HPr concentration-dependent kinetics catalyzed by the solubilized membranes and the open membrane structures (Fig. 3) in the high affinity regime seems to be consistent with the notion that the different environments most likely are restricted to the membrane-bound part of enzyme II^{mtl}, which is the interaction site for mannitol. This would leave the environment of the cytoplasmic domains of enzyme II^{mtl}, which constitute the phosphorylation pathway, identical under these conditions.

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