

Subunit Structure and Activity of the Mannitol-Specific Enzyme II of the *Escherichia coli* Phosphoenolpyruvate-Dependent Phosphotransferase System Solubilized in Detergent[†]

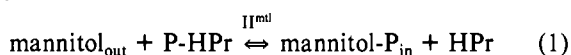
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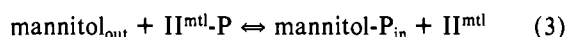
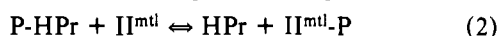
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ABSTRACT: The original proposal of Saier stating that P-enolpyruvate-dependent mannitol phosphorylation is catalyzed by the monomeric form of the bacterial phosphotransferase enzyme II^{mtl}, which would be the form predominantly existing in the phospholipid bilayer, whereas mannitol/mannitol-P exchange would depend on the transient formation of functional dimers, is refuted [Saier, M. H. (1980) *J. Supramol. Struct.* 14, 281-294]. The correct interpretation of the proportional relation between the rate of mannitol phosphorylation in the overall reaction and the enzyme concentration is that enzyme II^{mtl} is dimeric under the conditions employed. Differences measured in the enzyme concentration dependency of the overall and exchange reactions were caused by different assay conditions. The dimer is favored over the monomer at high ionic strength and basic pH. Mg²⁺ ions bind specifically to enzyme II^{mtl}, inducing dimerization. A complex formed by mixing inorganic phosphate, F⁻, and Mg²⁺ at sufficiently high concentrations inhibits enzyme II^{mtl}, in part, by dissociation of the dimer. Enzyme II^{mtl} was dimeric in 25 mM Tris, pH 7.6, and 5 mM Mg²⁺ over a large enzyme concentration range and under many different turnover conditions. The association/dissociation equilibrium was demonstrated in phosphate buffers, pH 6.3. The dimer was the most active form both in the overall and in the exchange reaction under the conditions assayed. The monomer was virtually inactive in mannitol/mannitol-P exchange but retained 25% of the activity in the overall reaction.

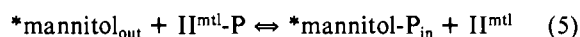
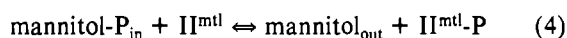
Transport of mannitol into *Escherichia coli* is catalyzed by enzyme II^{mtl},¹ which is part of the P-enolpyruvate-dependent phosphotransferase system (Postma & Lengeler, 1985; Robillard & Lolkema, 1988). Mannitol accumulates in the cell as mannitol-P; enzyme II^{mtl} couples transport and phosphorylation activity. The phosphoryl group donor is P-HPr, that itself is phosphorylated by P-enolpyruvate in a reaction catalyzed by enzyme I. Therefore, the overall reaction catalyzed by enzyme II^{mtl} is



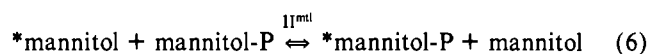
The reaction proceeds via the phosphorylated enzyme intermediate and, therefore, can be split into two partial reactions:



The latter of these predicts that enzyme II^{mtl} catalyzes equilibrium exchange between mannitol and mannitol-P through a similar mechanism:



Mannitol/mannitol-P exchange catalyzed by enzyme II^{mtl} solubilized in detergent:



can be readily demonstrated. Although there is general agreement that the overall reaction (reaction 1) proceeds

through reactions 2 and 3, there is less agreement that the exchange process (reaction 6) proceeds through the straightforward interpretation of eq 4 and 5 (see below).

Enzyme II^{mtl} has been purified to homogeneity. The purified enzyme is capable of mannitol phosphorylation when solubilized in the appropriate detergent, showing that a single polypeptide is sufficient for catalytic activity. In the past decade, data have accumulated demonstrating that the enzyme exists in at least two aggregation states, presumably monomer and dimer. The existence of dimeric enzyme II^{mtl} has been demonstrated by mild extraction followed by SDS-PAGE (Roossien & Robillard, 1984; Stephan & Jacobson, 1986), cross-linking studies (Roossien et al., 1986), radiation inactivation (Pas et al., 1987), size-exclusion chromatography (Khandekar & Jacobson, 1989), and stoichiometry of the enzyme-substrate complex (Pas et al., 1988). None of these techniques, however, provides a direct link between the activity of the enzyme and the subunit structure.

The relevance of subunit interactions within the dimer follows from kinetic measurements. In this respect, the original observation of Saier that started investigations into the association state of enzyme II^{mtl} is still the most important one to date. It demonstrated that mannitol/mannitol-P exchange activity (reaction 6) catalyzed by purified enzyme II^{mtl} solubilized in detergent increased quadratically with the enzyme concentration, strongly indicative of dimer formation. Surprisingly, the activity of enzyme II^{mtl} in the overall reaction (reaction 1) was proportional to the enzyme concentration. The data were confirmed in our laboratory (Roossien et al., 1984). The quadratic concentration dependence was also confirmed in the case of the exchange reaction for the purified enzyme reconstituted in proteoliposomes (Leonard & Saier,

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¹ Abbreviations: DTT, dithiothreitol; decylPEG, decylpoly(ethylene glycol) 300; mtl, mannitol.

1983). Saier proposed that the overall reaction was catalyzed by the monomer and the exchange reaction by the dimer. It was not recognized at that point that a proportional relation between activity and enzyme concentration does not allow any conclusion about the subunit structure. Later, a nonlinear relation between enzyme II^{mtl} activity in the overall reaction and enzyme concentration was reported using enzyme II^{mtl} purified with a modified procedure and in a different detergent (Robillard & Blaauw, 1987). It was argued that the higher steady state-level of phosphorylation of enzyme II^{mtl} in the overall reaction or the presence of residual phospholipid could promote dimerization. Unfortunately, most of the indirect evidence on the association state of enzyme II^{mtl} contradicts the proposed effect of phosphorylation on the monomer/dimer equilibrium (Stephan & Jacobson, 1986; Khandekar & Jacobson, 1989).

To clarify this important matter, we repeated and extended the original experiments of Saier. We will show that, although the experiments themselves were correct, comparison of the overall and exchange reaction led to a wrong conclusion because of the different conditions used in both reactions. Finally, we will clarify which factors, other than turnover conditions, affect the monomer/dimer equilibrium.

EXPERIMENTAL PROCEDURES

Materials

D-[1-³H(N)]Mannitol (706.7 GBq/mmol) was purchased from NEN Research Products. D-[1-¹⁴C]-Mannitol (2.2 GBq/mmol) was from Amersham. Decylpoly(ethylene glycol) 300 (decylPEG) was synthesized by B. Kwant in our laboratory. The *E. coli* phosphotransferase enzymes E_I and HPr were purified as described (Robillard et al., 1979; Dooijewaard et al., 1979).

Methods

Growth Conditions. *Escherichia coli* strain ML308-225 was grown at 37 °C in medium 63 (Saier et al., 1976) containing 0.5% mannitol as the carbon source. Cells were grown in 5-L flasks, filled with 2 L of medium, and aerated by continuous shaking. Cells were harvested at an OD₆₅₀ of 1.0.

Membrane vesicles were prepared essentially as described (Reenstra et al., 1980). The vesicles were washed once with 25 mM Tris, pH 7.6, 1 mM DTT, and 1 mM Na₂N₃. Aliquots of 50 μL containing 1 mg/mL membrane protein (Lowry et al., 1951) were stored in liquid nitrogen. Samples were thawed rapidly before use. Each sample was used only 1 time.

Enzyme II^{mtl} was purified from these membranes as described (Roossien et al., 1984) with modifications (Robillard & Blaauw, 1987).

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 constituents are stated in the figure legends. Four samples of 20 μL each were withdrawn at consecutive times and analyzed for labeled mannitol-P as described (Robillard & Blaauw, 1987). A fifth sample of 10 μL was used to relate the labeled mannitol concentration to the total radioactivity in the sample. The mannitol/mannitol-P exchange reaction was initiated by adding 20 μL of a mannitol-P solution to 80 μL of enzyme suspension incubated with labeled mannitol. Routinely, the P-enolpyruvate-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 EI. The latter requires Mg²⁺ as a cofactor. Some experiments, however, require very low concentrations of Mg²⁺. In these cases, the overall reaction was initiated by P-HPr, synthesized in situ in the following way. An appropriate concentration of HPr was incubated for 10 min at 30 °C with 5 mM P-enolpyruvate, 0.22 μM enzyme I, and 0.125 μM MgSO₄. In parallel, the solubilized enzyme II^{mtl} was incubated with labeled mannitol in the absence of Mg²⁺. The reaction was initiated by adding 20 μL of the P-HPr solution to 80 μL of the enzyme suspension, making the final Mg²⁺ concentration 25 μM. The proper functioning of the assay was tested by adding a solution of 7 μM P-HPr, synthesized in this way, to enzyme II^{mtl} incubated with 0.4 μM [³H]mannitol in 100 mM phosphate buffer, pH 6.3, with 1 mM EDTA or with 10 mM MgSO₄. The initial rate of mannitol phosphorylation was identical in both experiments.

RESULTS

Different specific activities of monomeric and dimeric enzyme in a dynamic association/dissociation equilibrium show up when the activity is measured as a function of the enzyme concentration. Mass action predicts an increase of dimers over monomers with increasing total concentration. The fraction of dimers increases quadratically only if its fraction is very small. This is the basis of the detection of a monomer/dimer equilibrium with different specific activities for monomer and dimer that we have used throughout this paper.

Specific Activity of Enzyme II^{mtl} in Tris/Mg²⁺, pH 7.6, Buffer. The phosphoenolpyruvate-dependent phosphorylation of mannitol catalyzed by cytoplasmic membranes solubilized in the detergent decylPEG was measured as a function of membrane concentration in a buffer containing 25 mM Tris, pH 7.6, and 5 mM MgSO₄ (Figure 1). The experiment was repeated under a wide variety of turnover conditions to anticipate possible effects of steady-state levels of phosphorylated enzyme or mannitol-enzyme complex. The filled symbols in Figure 1 show the rate of mannitol phosphorylation assayed over a thousandfold mannitol concentration range with saturating concentrations of P-HPr. The steady-state level of phosphorylation of enzyme II^{mtl} was lowered by reducing the P-HPr concentration (see reactions 1 and 2; Figure 1, open symbols). The rate of mannitol phosphorylation was proportional to the membrane concentration under all these conditions.

The source of enzyme II^{mtl} in the above experiments was solubilized membranes. However, a number of these experiments were repeated with purified enzyme II^{mtl} in the detergent decylPEG with identical results. For instance, the rate of P-enolpyruvate-dependent mannitol phosphorylation catalyzed by purified enzyme II^{mtl} was measured with 40 μM mannitol and 3 μM HPr in the buffer described in the legend of Figure 1. The phosphorylation rates were measured in ranges of 10–50 pM and 0.5–2.5 nM enzyme II^{mtl}. In both cases, the rate was proportional to the enzyme concentration with essentially the same slopes, 620 and 680 min⁻¹, respectively (not shown). Therefore, the specific activity of purified enzyme II^{mtl} in the overall reaction is constant over a range of enzyme concentrations from 10 pM to 2.5 nM.

Comparison of the mannitol phosphorylation activity catalyzed by solubilized membranes and purified enzyme under exactly the same conditions allows for a fair estimate of the mannitol permease concentration in the cytoplasmic membranes used above. The concentration amounts to 400 nM enzyme II for a 1 mg/mL membrane protein suspension. Consequently, the enzyme II^{mtl} concentration in Figure 1

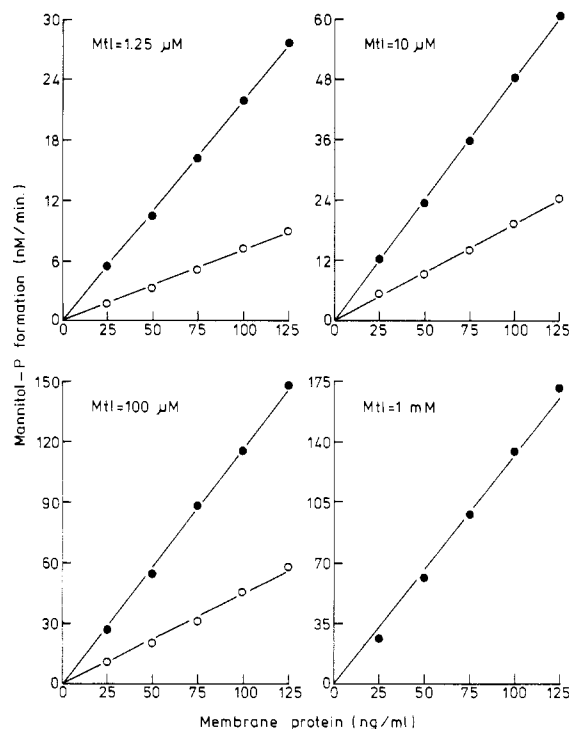


FIGURE 1: Relation between the activity of enzyme II^{mII} in the overall reaction and the enzyme concentration under different turnover conditions. Mannitol phosphorylation catalyzed by solubilized membranes was measured in 25 mM Tris, pH 7.6, 5 mM MgSO₄, 5 mM DTT, and 0.25% decylPEG. The phosphorylation activity was measured at the indicated mannitol concentrations, both at saturating (●) and at subsaturating (○) concentrations of HPr. The HPr concentrations in the experiments with 1.25 μM mannitol were 3 and 0.25 μM; with 10 μM mannitol, 4 and 0.5 μM; with 100 μM mannitol, 24 and 2 μM; with 1 mM mannitol, 24 μM.

ranges from 10 to 50 pM. When the activity was measured at the lower detection limit, where the membrane protein concentration was varied from 0.25 to 1.25 ng of membrane protein/mL (100–500 fM enzyme II), a specific activity of 183 nM/(min·mg) was found (not shown). This was similar to the value of 156 nM/(min·mg) estimated from the phosphorylation rate catalyzed by a hundredfold higher membrane protein concentration (125 ng/mL) under identical conditions. Therefore, extremely low enzyme concentrations do not significantly affect the specific activity.

The mannitol/mannitol-P exchange activity was measured as a function of the enzyme concentration in the same Tris/Mg²⁺, pH 7.6, buffer (Figure 2). In contrast to all earlier reports, the specific activity was independent of the enzyme concentration. The conditions used were different from the earlier experiments, but exactly the same as in the above experiments. There seem to be no difference in the enzyme concentration dependence of the overall versus exchange reaction if measured under identical conditions. Different mannitol or mannitol-P concentrations do not affect the linearity. The low activity at 4 μM mannitol can be explained by the well-documented inhibition of mannitol in the exchange reaction.

In conclusion, the experiments presented thus far do not indicate any involvement of an association/dissociation equilibrium in the catalytic activities of enzyme II^{mII} in the overall reaction or in the exchange reaction. No statement can be made about the subunit structure, if any, of the per-mease.

Inhibition of Enzyme II^{mII} in Phosphate Buffers. The pH optimum for the exchange reaction is more acidic than for the overall reaction. Consequently, exchange activities have been

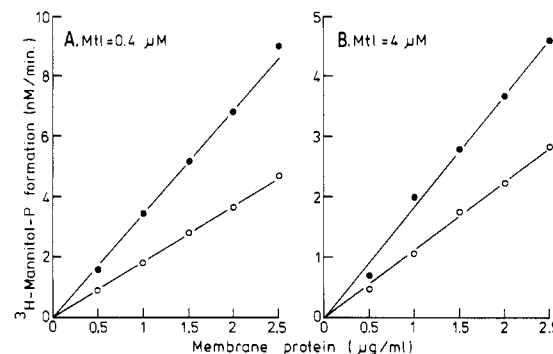


FIGURE 2: Relation between the activity of enzyme II^{mII} in the mannitol/mannitol-P exchange reaction and the enzyme concentration under different turnover conditions. Mannitol/mannitol-P exchange catalyzed by solubilized membranes was measured in 25 mM Tris, pH 7.6, 5 mM MgSO₄, 5 mM DTT, and 0.25% decylPEG. The exchange activity was measured with 0.4 μM (A) mannitol and 4 μM (B) mannitol. The concentrations of mannitol-P were (A) 4 mM (●) and 1 mM (○) and (B) 4 mM (●) and 2 mM (○).

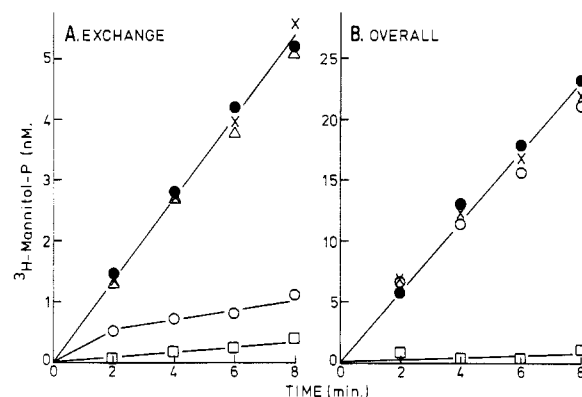


FIGURE 3: Inhibition of enzyme II^{mII} by a complex of P_i, Mg²⁺, and F⁻ in the exchange and the overall reaction. Membranes (0.25 μg/mL) were incubated in 25 mM Tris, pH 7.6, 5 mM MgSO₄, 5 mM DTT, and 0.25% decylPEG with 0.2 μM [³H]mannitol in a total volume of 60 μL. Two minutes before the assay was initiated, 20 μL of H₂O (●, Δ, ○), 50 mM NaF (×), or 50 mM NaF, 5 mM MgSO₄, and 10 mM P_i (□) were added. (A) Exchange reaction. At *t* = 0, an additional 20 μL of 10 mM mannitol-P (●, ×, □) was added together with 50 mM NaF (Δ) or 50 mM NaF plus 5 mM MgSO₄ (○). (B) Overall reaction. At *t* = 0, 20 μL of a mixture of 0.75 μM HPr, 10 mM P-enolpyruvate, and 0.22 μM enzyme I was added (●, ×, □). With experiment (○), this mixture, in addition, contained 50 mM NaF.

customarily assayed in phosphate buffers, pH 6.0–6.3. In addition, the buffers contained Mg²⁺, and NaF was added to inhibit putative phosphatase activity. This buffer composition appears to be inhibitory to enzyme II^{mII} due to the formation of a complex between P_i, F⁻, and Mg²⁺. Figure 3A shows the formation of [³H]mannitol-P in the exchange reaction in 25 mM Tris, pH 7.6, 5 mM MgSO₄, 5 mM DTT, and 0.25% decylPEG. A 5-fold dilution of a mixture of 10 mM P_i, 5 mM MgSO₄, and 50 mM NaF into the assay mixture just before the zero time point led to a rapid inhibition of the activity (□). Importantly, mannitol-P could replace P_i in this mixture (○). The formation of a complex between NaF, MgSO₄, and P_i or mannitol-P causing the inhibition was demonstrated by the necessity of preincubating the three components together at a sufficiently high concentration for the inhibition to occur. Adding one of them separately [Figure 3A Δ and ×] did not lead to inhibition even though the final composition of the buffer was the same.

The inhibition was not limited to the exchange reaction but could be demonstrated in the overall reaction as well (Figure 3B). P-Enolpyruvate did not seem to be able to replace P_i in

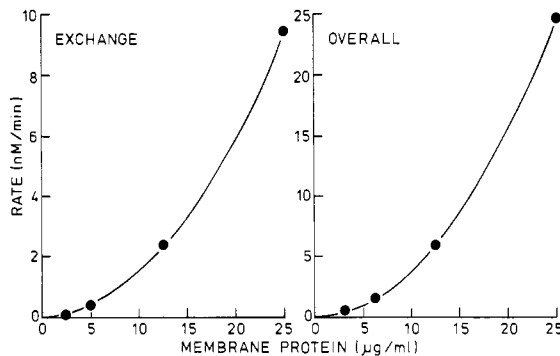


FIGURE 4: Nonlinear relation between enzyme concentration and activity in the exchange and overall reaction under inhibitory conditions. Solubilized membranes were incubated in 25 mM Tris, pH 7.6, 5 mM MgSO_4 , 5 mM DTT, and 0.25% decylPEG with 0.4 μM [^3H]mannitol. A 20- μL mixture of 50 mM NaF, 5 mM MgSO_4 , and 10 mM P_i was added to a volume of 60 μL . The exchange reactions were initiated with 20 μL of 20 mM mannitol-P. The overall reaction was initiated by addition of a mixture of 180 nM HPr, 5 mM P-enolpyruvate, 1 mM MgSO_4 , and 110 nM enzyme I.

the inhibitory complex (O). Phosphorylated enzyme II^{ml} was protected against the inhibition relative to the unphosphorylated enzyme. Preincubating the enzyme with a high concentration of P-HPr prior to the addition of the inhibitory complex resulted in normal activity at first that rapidly decreased due to the continuous dephosphorylation of the enzyme during turnover (not shown).

The exchange activity measured as a function of the enzyme concentration under these inhibitory conditions increased rapidly with the enzyme concentration. Similar behavior was observed for enzyme II^{ml} in the overall reaction (Figure 4). The nonlinear relations do not automatically prove that the inhibitory complex dissociates associated enzyme II^{ml} . Since we do not know the concentration of the inhibitory complex, it could well be of the same order of magnitude as the enzyme concentration. Then, the phenomenon could be explained by a strong binding between inhibitor and enzyme. All enzyme would be in the complexed, inhibited state when the K_D for binding is much lower than the enzyme and inhibitor concentrations. However, a sudden release of inhibition would be apparent when the enzyme concentration starts exceeding the inhibitor concentration. Increasing enzyme concentrations would titrate the inhibitor out of solution. A more than quadratic increase of the activity with the enzyme concentration often seen in these experiments is an indication of such a process. On the other hand, when membrane-bound enzyme II^{ml} was used, inhibition was less than a factor of 2, and there was no clear effect of the membrane concentration or inhibitor concentration (not shown). Since the permease concentration is a constant within each membrane particle and mass action is restricted, this observation is in line with a mechanism of dissociation by the inhibitor when the enzyme is solubilized in detergent. Of course, both mechanisms could be acting together.

Association/Dissociation of Enzyme II^{ml} . Stephan and Jacobson (1986) have demonstrated a higher exchange activity in 100 mM phosphate buffer compared to 100 mM HEPES buffer. It was suggested that inorganic phosphate promotes dimerization of enzyme II^{ml} [see also Khandekar and Jacobson (1989)]. Figure 5 demonstrates the activity of enzyme II^{ml} in the overall reaction and the exchange reaction in phosphate buffer, pH 6.3, as a function of the buffer concentration. The exchange activity (Figure 5B) was sigmoidal, starting at very low activity in 10 mM phosphate and reaching a maximum activity at around 75 mM (●). Small concentrations of

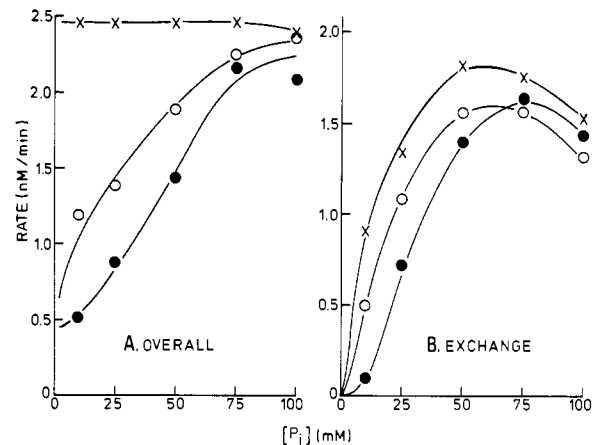


FIGURE 5: Activity of enzyme II^{ml} as a function of the buffer concentration and the Mg^{2+} concentration. Membranes (50 ng/mL and 0.5 $\mu\text{g/mL}$ for overall and exchange, respectively) were solubilized in phosphate buffer, pH 6.3, 5 mM DTT, and 0.25% decylPEG. (A) Overall reaction. The activity was measured by using 0.2 μM [^3H]mannitol, 1.4 μM HPr, 22 nM enzyme I, and 1 mM P-enolpyruvate. The MgSO_4 concentrations were 25 μM (●), 1 mM (○), and 10 mM (×). (B) Exchange reaction. The activity was measured by using 0.4 μM [^3H]mannitol and 1 mM mannitol-P. MgSO_4 concentrations: none (●), 50 μM (○), and 500 μM (×).

Table I: Effect of Ionic Strength and Mg^{2+} Ions on the Activity of Enzyme II^{ml}

addition	exchange rate [nmol min ⁻¹ (mg of membrane protein) ⁻¹]	
	10 mM P_i	75 mM P_i
none	0.17	3.5
87.5 mM KCl	3.3	2.8
87.5 mM NaF	2.1	2.2
37.5 mM Na_2SO_4	3.6	3.2
10 mM MgCl_2	3.1	3.2
0.5 mM MgCl_2	1.6	
0.5 mM MgSO_4	1.6	
0.5 mM K_2SO_4	0.15	

^a The rate of exchange between 0.2 μM [^3H]mannitol and 1 mM mannitol-P catalyzed by solubilized membranes (0.5 $\mu\text{g/mL}$) was measured in 10 or 75 mM phosphate buffer, pH 6.3, 5 mM DTT, and 0.25% decylPEG.

MgSO_4 stimulated the activity at low phosphate concentrations with little effect at the higher concentrations, indicating that the mechanism of stimulation by inorganic phosphate and MgSO_4 is the same (O, ×). These findings were qualitatively identical with the overall reaction (Figure 5A). The phosphoryl group donating substrate, P-HPr, was made in situ just before the start of the measurements (see Methods). The activity in 10 mM phosphate buffer is higher (●) than the activity in the exchange reaction for reasons that will be explained later. Again, higher concentrations of MgSO_4 stimulated the activity only at the lower enzyme concentrations (O); in 10 mM MgSO_4 , the activity became independent of the phosphate concentration (×).

Table I demonstrates that the stimulation of the activity by inorganic phosphate is aspecifically caused by an increase of the ionic strength of the solution. Mg^{2+} ions, on the other hand, exert their stimulation in a more specific way. No effect on the activity was seen when several salts were added to 75 mM phosphate buffer. A strong stimulation, on the other hand, was seen upon the addition of the same salts to 10 mM phosphate buffer. The salts stimulated the activity up to the value found in 75 mM phosphate buffer. NaF seemed to have some inhibitory effect at these high concentrations as it reduced the activity somewhat in 75 mM phosphate and showed less

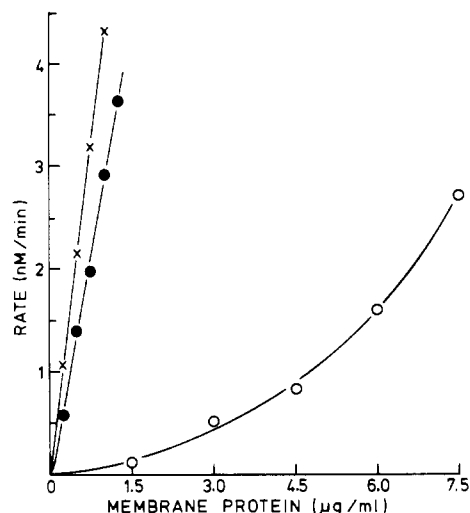


FIGURE 6: Dissociation of enzyme II^{mtl} in buffers of low ionic strength in the absence of Mg²⁺. Exchange activity was measured in 10 mM P_i (○), 100 mM P_i (●), and 10 mM P_i plus 5 mM MgSO₄ (×). The pH was 6.3. In addition, the buffers contained 5 mM DTT and 0.25% decylPEG and the indicated concentrations of membrane protein. Assay conditions: 0.4 μM mannitol and 1 mM mannitol-P.

stimulation in 10 mM phosphate. Both 0.5 mM MgSO₄ and MgCl₂ promoted the same strong stimulation in 10 mM phosphate, indicating the specificity for Mg²⁺. The stimulation is not due to an increase in ionic strength since 0.5 mM Na₂SO₄, whose contribution to the ionic strength is identical with 0.5 mM MgCl₂, did not stimulate significantly. Therefore, enzyme II^{mtl} possess a Mg²⁺ binding site.

Figure 6 shows the mannitol/mannitol-P exchange activity as a function of the enzyme concentration in 10 mM phosphate buffer (○). The relation was quadratic in this low ionic strength buffer, typical for the formation of active dimers. In contrast, the relation was linear in 100 mM P_i (●) or when 5 mM MgSO₄ was added to the 10 mM P_i buffer (×). The much lower activity in buffers of low ionic strength in the absence of Mg²⁺ seems to be due to dissociation of the enzyme. Both high ionic strength and Mg²⁺ promote dimerization of the enzyme. The specific activity of the dimer in the presence of Mg²⁺ (×) is higher than observed in 100 mM P_i (●), which is due to inhibition by the high phosphate concentration, also evident from Figure 5B.

Apparently, monomeric enzyme II^{mtl} is much less active in the mannitol/mannitol-P exchange reaction. This is different from the activity of monomeric enzyme II^{mtl} in the overall reaction. Figure 7 (×) shows that the specific activity in the overall reaction is rather constant when assayed over a 125-fold concentration range in 10 mM phosphate and 25 μM MgSO₄. The specific activity readily reaches a 4–5 times higher level when 10 mM MgSO₄ is added (●). In the intermediate case [1 mM MgSO₄ (○)], the specific activity goes from the lower curve to the higher. The insensitivity of the lower curve (×) to the enzyme concentration indicates that, in this concentration range, all enzyme is monomeric. Therefore, the monomer has retained some 25% of its activity in the overall reaction whereas the monomer is essentially inactive in the exchange reaction. In conclusion, modulation of enzyme II^{mtl} activity by ionic strength and Mg²⁺ concentration demonstrates the dynamic equilibrium between monomeric and dimeric enzyme II^{mtl}. Monomeric enzyme II^{mtl} is much more inhibited in the mannitol/mannitol-P exchange reaction than in the P-enolpyruvate-driven reaction. The ionic strength induced dimerization indicates that the dimer is stabilized by hydrophobic interactions.

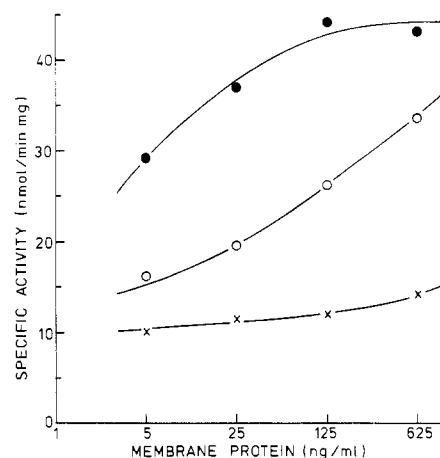


FIGURE 7: Effect of association/dissociation of enzyme II^{mtl} on the specific activity in the overall reaction. The activity of solubilized membranes was measured in 10 mM P_i, pH 6.3, 5 mM DTT, 0.25% decylPEG, and 25 μM (×), 1 mM (○), or 10 mM (●) MgSO₄. [³H]Mannitol concentration: 0.2 μM. The reaction was initiated by addition of 7 μM P-HPr. Membrane protein concentration was plotted on a logarithmic scale.

DISCUSSION

Differences reported in the past between the behavior of enzyme II^{mtl} when catalyzing P-enolpyruvate-dependent mannitol phosphorylation or mannitol/mannitol-P exchange with respect to association/dissociation of the enzyme were caused by different conditions used in the assays. The present data show that the monomer/dimer equilibrium is affected by the ionic strength, Mg²⁺ ions, pH, and a complex between P_i, F⁻, and Mg²⁺.

Ionic Strength. The effects of inorganic phosphate reported by the lab of Jacobson (Stephan & Jacobson, 1986; Khandekar & Jacobson, 1989) can be generalized to effects of the ionic strength of the solution. Table I shows that any salt is capable of stimulating the exchange activity. The link between this effect and the association state of enzyme II^{mtl} is evident from the relation between activity and enzyme concentration in buffers of high and low ionic strength (Figure 6). The lower activity in the low ionic strength buffer could be compensated for by an increase in the enzyme concentration, indicating association. All enzyme is dimeric in the same enzyme concentration range at high ionic strength. Hydrophobic interaction seems to be the driving force for the association.

Mg²⁺ Ions. The effect of Mg²⁺ ions on the association state of enzyme II^{mtl} is identical with the other salts, except for the fact that it exerts its action at a much lower concentration (Table I, Figures 6 and 7). Therefore, Mg²⁺ binds specifically to enzyme II^{mtl}, causing the association equilibrium to shift to the dimeric form. Mg²⁺ is not essential for activity of enzyme II^{mtl}. Normal phosphorylation rates are measured in buffers of high ionic strength in the absence of Mg²⁺ (e.g., Table I) or even in the presence of EDTA (see Activity Measurements under Methods).

pH. The specific activity of enzyme II^{mtl} in 25 mM Tris, pH 7.6, and 5 mM MgSO₄ is not reduced at membrane protein concentrations as low as 0.25 ng/mL. On the other hand, a significant fraction of monomers is present in 10 mM P_i, pH 6.3, 10 mM MgSO₄, and 5 ng/mL membrane protein [Figure 7 (●)]. This suggests that dimers are favored over monomers at more basic pH.

Complex of P_i, F⁻, and Mg²⁺. The inhibitory effect of the complex is, most likely, at least in part due to dissociation of the enzyme. The formation of the complex requires the presence of all three components, though P_i may be replaced

by mannitol-P. At high concentrations, a precipitate is formed. The unknown character of the complex makes it desirable to avoid its formation in the assays.

The behavior of enzyme II^{mtl} when catalyzing the overall reaction or the exchange reaction is identical under identical conditions: (i) In Tris/Mg²⁺, pH 7.6, buffer where enzyme II^{mtl} is a dimer, the activity increases proportionally with the enzyme concentration both in the exchange and in the overall reaction (Figures 1 and 2). (ii) Under the inhibitory conditions in the presence of the complex between P_i, F⁻, and Mg²⁺, the activity increases more than proportionally with the enzyme concentration in both reactions. Monomeric enzyme seems to be inactive both in the exchange and in the overall reaction under these conditions (Figure 4). (iii) Both specific activities increase with the enzyme concentration in buffers of low ionic strength and low Mg²⁺ concentration. The dimer is the more active form. Although the monomer has retained some 25% of its activity in the overall reaction, it seems to have lost essentially all of the activity in the exchange reaction. Clearly, in the past, condition i has been used for the overall reaction and condition ii or iii for the exchange reaction. The different behavior was explained at the level of the mechanism of the two reactions; different association states of enzyme II^{mtl} would be the catalytic units catalyzing P-enolpyruvate-dependent phosphorylation or mannitol/mannitol-P exchange. The present data make it unnecessary to propose a different mechanism for the exchange reaction. Equations 4 and 5 in the introduction are likely to be correct in describing the mechanism by which exchange proceeds.

Dimeric enzymes fall into three classes where the relation of the quaternary structure with the activity is concerned: (I) Structural dimers. Dimerization has no effect on the activity. Each monomer within the dimer is the same catalytic unit as in the dissociated state. (II) Catalytic dimer. The dimer is the catalytic unit. Sites on both monomers within the dimer contribute to the catalytic center (shared sites). The monomer by itself is incomplete and inactive. (III) Cooperative dimer. In the intermediate case, each monomer within the dimer is a complete catalytic unit, but the two affect one another, positively or negatively. The specific activity of monomer and dimer is different.

The results with the exchange reaction might suggest that enzyme II^{mtl} falls in class II. However, the lower but significant activity of the monomer in the overall reaction clearly shows that this should be class III. The monomer, by itself, possesses the machinery (P-HPr and mannitol binding sites, phosphoryl group binding sites) to phosphorylate mannitol with P-HPr as phosphoryl group donor. Consequently, the monomer should also be able to catalyze mannitol/mannitol-P exchange, since this is a partial reaction of the overall reaction (eq 3). If it does not, as seem to be the case, it is kinetically inhibited. This inhibition may be related to the strong substrate inhibition observed in the dimer-catalyzed exchange reaction (compare panels A and B of Figure 2). We have proposed that the mechanism of this inhibition is competition between mannitol and mannitol-P for the binding site on the enzyme (Robillard & Lolkema, 1988). Binding of mannitol would prevent the phosphorylation of enzyme II^{mtl} by mannitol-P. This mechanism may be more severe when only one binding site is available in the monomer versus two in the dimer.

Kinetic analysis of mannitol phosphorylation catalyzed by monomeric and dimeric enzyme II^{mtl} will reveal the interaction between the two subunits within the dimer. Preliminary experiments indicate that the specific activity of the monomer

relative to the dimer depends on turnover conditions. The conditions used in this study were chosen such that the monomer/dimer equilibrium could be demonstrated in the overall reaction. The kinetic characteristics of monomer and dimer should not be confused with effects upon the monomer/dimer equilibrium itself. Khandekar and Jacobson (1989) have demonstrated that enzyme II^{mtl} solubilized in the detergent deoxycholate eluted at the dimer position from a molecular sizing column. Phosphorylation of the enzyme or the presence of extremely high concentrations of mannitol shifted the enzyme to the position of a monomer. Our data do not indicate strong effects of the turnover conditions (Figures 1 and 2) even if the steady-state degree of phosphorylation of enzyme II^{mtl} is high and the enzyme concentration low. Of course, these effects may be overruled by the stronger effects of the buffer composition or the type of detergent. It may be of relevance that the enzyme is inactive in 0.25% deoxycholate. Experiments are in progress to settle this apparent discrepancy.

Finally, this paper treats only the relation between subunit structure and mannitol phosphorylation activity; this does not necessarily include transport activity. Transport has lost its meaning in detergent solutions. It is conceivable that transport does require dimeric enzyme II^{mtl}, e.g., to form a translocation site at the interface of the two subunits. Given the conditions that favor dimerization and the high concentration of the enzyme in the two-dimensional space of the membrane, it is likely that enzyme II^{mtl} embedded in the membrane is predominantly dimeric.

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