Mannitol-Specific Carrier Protein from the *Escherichia coli* Phosphoenolpyruvate-Dependent Phosphotransferase System Can Be Extracted as a Dimer from the Membrane†

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ABSTRACT: The association state of the mannitol-specific enzyme II (EII^{Mtl}) has been studied both in the purified form and embedded in the cytoplasmic membrane. Membrane fragments obtained from mannitol-grown *Escherichia coli* catalyze the phosphoenolpyruvate- (PEP) dependent phosphorylation of both glucose and mannitol; thus they contain both the glucose- and mannitol-specific enzymes II. The autoradiogram of an electrophoresed mixture of [^{32}P]PEP, EI, HPr, and membrane fragments shows bands at 58 and 116 kilodaltons, in addition to the bands of P-EI and P-HPr. In an analogous experiment with purified EII^{Mtl}, suspended in detergent micelles, only a 58 000-dalton band and the P-HPr and P-EI bands were found. Treatment of the phosphorylated membranes with mannitol results in an immediate substantial decrease in the radioactivity in the 58- and 116-kilodalton bands. A similar treatment of the phosphorylated membranes with glucose had no direct effect on the autoradiogram. We conclude therefore that the 58- and 116-kilodalton bands originate from enzyme II^{Mtl} monomers and dimers, respectively. The interaction between the subunits of the dimer is not abolished by the addition of up to 5% sodium dodecyl sulfate. However, the nonionic detergent Lubrol PX, which is present during the purification of EII^{Mtl}, is capable of transforming the enzyme II^{Mtl} dimers into monomers.

The bacterial phosphoenolpyruvate- (PEP)[1] dependent phosphotransferase system (PTS) catalyzes the concomitant transport and phosphorylation of a number of hexoses and hexitols. The mannitol-specific PTS consists of two cytoplasmic proteins EI and HPr and an integral membrane protein, enzyme II (EII^{Mtl}). Scheme I shows the sequence of reactions that lead to the transfer of the phosphoryl group of PEP to mannitol.

Scheme I

$$\text{PEP} + \text{EI} \rightleftharpoons \text{P-EI} + \text{pyruvate}$$

$$\text{HPr} + \text{P-EI} \rightleftharpoons \text{P-HPr} + \text{EI}$$

$$\text{P-HPr} + EII^{Mtl} \rightleftharpoons \text{P-}EII^{Mtl} + \text{HPr}$$

$$\text{mannitol} + \text{P-}EII^{Mtl} \rightleftharpoons EII^{Mtl} + \text{mannitol-P}$$

In addition to the PEP-dependent mannitol phosphorylation, EII^{Mtl}, in the absence of EI and HPr, catalyzes the exchange reaction shown in Scheme II (Saier, 1980).

Scheme II

$$[^{14}\text{C}]\text{mannitol} + \text{mannitol-P} \xrightleftharpoons{EII^{Mtl}} \text{mannitol} + [^{14}\text{C}]\text{mannitol-P}$$

Jacobson et al. (1979) developed an isolation procedure for EII^{Mtl} that yielded a homogeneous preparation with a molecular weight in the range of 60 000.

Kinetic studies with purified EII^{Mtl} showed that the exchange reaction proceeds via an oligomeric (possibly dimeric) form of the protein (Leonard & Saier, 1983; Roossien et al., 1984). Our recent studies on the dithiol disulfide distribution in the purified enzyme have shown that a dithiol is essential for enzymatic activity. When it is exposed to oxidizing conditions, a disulfide is formed. The disulfide is an intersubunit disulfide supporting the dimeric nature of the enzyme (Roossien & Robillard, 1984).

In this paper we show that EII^{Mtl} can be extracted as a dimer from the cytoplasmic membrane. Recently Goldkorn et al. (1984) showed that the functional mass of the integral membrane *lac* carrier protein from *Escherichia coli* changes from a monomer into a dimer upon energization of the membrane. Dimerization appears to be of importance in the EII^{Mtl}-catalyzed reactions as well.

Experimental Procedures

[γ-^{32}P]ATP (2×10^4 Ci/mol) and [^{14}C]PEP (10.6 mCi/mmol) were purchased from Amersham. [^{32}P]PEP (specific activity 7×10^3 cpm/pmoi) was prepared from [^{32}P]ATP by the method of Roossien et al. (1983).

EI and HPr were purified as described previously by Dooyewaard et al. (1979) and Robillard et al. (1979). EI and HPr concentrations were determined with the [^{14}C]pyruvate burst method described by Brouwer et al. (1982). EII^{Mtl} was purified according to the procedure of Jacobson et al. (1979) including the modifications described by Roossien & Robillard (1984).

Cytoplasmic Membranes. Escherichia coli ML308/225 was grown under aerobic conditions at 37 °C in medium 63 (Saier et al., 1976) containing 0.5% mannitol as the carbon source. Inside-out and right-side-out vesicles were prepared as described by Reenstra et al. (1980) and Kaback (1971), respectively. Membranes were prepared from inside-out vesicles as described by Jacobson et al. (1979) or from right-side-out vesicles by passage through a French press (10 000 psi) at 4 °C. Membranes were stored in liquid nitrogen until used.

Solubilization and Electrophoresis. Protein samples were diluted with an equal volume of solubilization buffer, leading to a final concentration of 62.5 mM Tris-HCl, pH 8.8, 2% SDS, 5% mercaptoethanol, and 10% glycerol. Unless other-

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[1] Abbreviations: PEP, phosphoenolpyruvate; EII^{Mtl}, mannitol-specific enzyme II; EII^{Man}, mannose-specific enzyme II; EII^{Glc}, glucose-specific enzyme II; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)-aminomethane; PAGE, polyacrylamide gel electrophoresis; DOC, deoxycholate; DTT, dithiothreitol; EI, enzyme I; PTS, phosphotransferase system.

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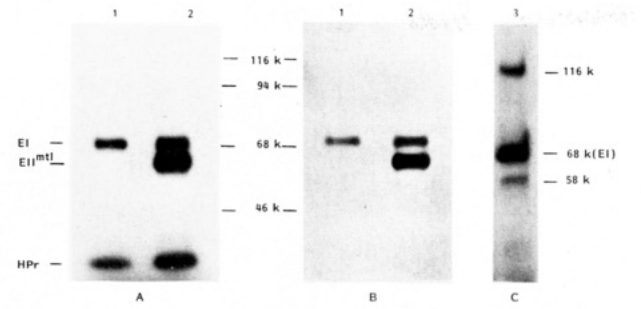

FIGURE 1: SDS gel electrophoresis and autoradiography of ^{32}P-labeled PTS components EI, HPr, and EII^{Mtl}. The proteins were phosphorylated at 30 °C in a mixture (final volume 25 μL) containing 0.5 mM $MgCl_2$, 1.0 mM NaF, 2.0 mM DTT, and 50 mM sodium phosphate buffer, pH 7.0. Sample 1 contained 1.0 μM HPr and 0.5 μM EI. Sample 2, in addition, contained 1.3 μM pure EII^{Mtl}. The (estimated) Lubrol PX concentration in sample 2 was 0.02%. Sample 3 had the same composition as sample 1 except that a cytoplasmic membrane preparation was added (final concentration 0.4 mg/mL) instead of purified EII^{Mtl}. At $t = 0$ [^{32}P]PEP was added to a final concentration of 12.5 μM. After 3 min the phosphorylation reaction was terminated by the addition of 25 μL of solubilization buffer, and electrophoresis was performed as described under Experimental Procedures. Part A shows the autoradiogram of samples 1 and 2. Part B shows the protein pattern of the gel after silver staining. Part C shows the autoradiogram of sample 3.

wise indicated, samples (30–80 μL) were incubated for 30 min at 30 °C before they were loaded carefully onto a 0.14 cm thick SDS–8% PAGE slab gel, prepared as described by Laemmli (1970) but with the stacking gel omitted. Electrophoresis was performed as described by Laemmli (1970). Thereafter, the gel was washed for 30 min in 1 L of electrophoresis buffer and autoradiographed on Kodak XAR-5 film for 10–30 h at 4 °C. The protein pattern of the gel was determined by the silver staining procedure described by Wray et al. (1981).

Results

Demonstration of Phosphorylated Intermediates of EI, HPr, and Purified EII^{Mtl}. The experiment presented in Figure 1 demonstrates that the phosphorylated PTS protein intermediates P-HPr, P-EI, and P-EII^{Mtl} can be detected on autoradiograms of SDS–polyacrylamide gels. Two samples were electrophoresed. The sample in lane 1 of parts A and B of Figure 1 contained purified HPr, EI, and [^{32}P]PEP. The sample in lane 2 contained purified EII^{Mtl} in addition to HPr, EI, and [^{32}P]PEP. The autoradiogram, Figure 1A, contains bands at 68 000 and 10 000 daltons corresponding to P-EI and P-HPr (lane 1) and, in addition, a band at 58 000 daltons when EII^{Mtl} is present (lane 2). After the autoradiogram was made, the gel was subjected to silver staining. The protein pattern, Figure 1B, shows bands at identical positions for the high molecular weight components. The protein band for HPr is not observable probably because HPr diffused out of the gel during the 18-h washing procedure prior to silver staining. When an EII^{Mtl}-containing membrane preparation was added in place of purified EII^{Mtl}, the autoradiogram (Figure 1C) showed phosphorylated components at 68 000 and 58 000 daltons corresponding to EI and EII^{Mtl}, respectively, and a component at 116 000 daltons. The presence of EI and HPr is mandatory for the appearance of this band. The remainder of this paper examines the identity of the 116 000-dalton component. Although in all membrane preparations the 58 000 and 116 000 bands were present in substantial amounts, the relative intensities of these bands sometimes differed. This probably originates from minor differences in the solubilization procedure, i.e., incubation time or protein–detergent ratio.

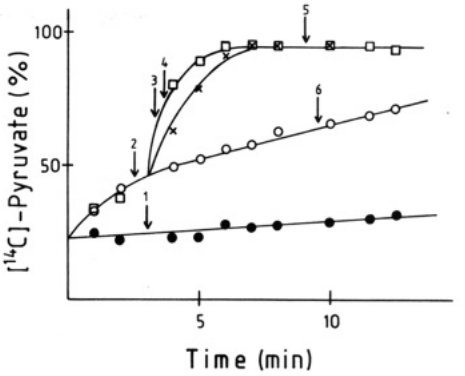

FIGURE 2: PTS-dependent [^{14}C]pyruvate production. The experiments were performed at 30 °C in a phosphorylation buffer containing the following components: 50 mM sodium phosphate buffer, pH 7.0, 0.5 mM $MgCl_2$, 2.0 mM NaF, 2.0 mM DTT, 1.0 μM HPr, and 0.5 μM EI. At $t = 0$ [^{14}C]PEP (12.5 μM) was added to samples that contained (a, ●) no extra additions, (b, ○) cytoplasmic membranes (0.4 mg/mL), (c, □) cytoplasmic membranes (0.4 mg/mL) to which 0.29 mM mannitol was added at $t = 3$ min, and (d, ×) membranes (0.4 mg/mL) to which 50 μM glucose was added at $t = 3$ min. At the indicated time intervals samples were withdrawn. The amount of [^{14}C]pyruvate formed is expressed as a percentage of the original [^{14}C]PEP concentration. The arrows with numbers refer to the times at which samples were removed for the gel electrophoresis presented in Figure 3A.

Demonstration of Membrane-Bound Enzyme II. Addition of [^{14}C]PEP to a mixture containing purified HPr and EI results in a burst of [^{14}C]pyruvate due to the instantaneous formation of P-HPr and P-EI. This behavior is shown in Figure 2 (●). The subsequent slow increase in [^{14}C]pyruvate is due to the slow hydrolysis of these phosphoproteins and their rephosphorylation. If *E. coli* cytoplasmic membrane fragments are present along with HPr and EI at the time of addition of [^{14}C]PEP, three phases in [^{14}C]pyruvate production are apparent (0–0): at first the initial burst observed with HPr and EI alone, then a second slower phase due to the phosphorylation of the membrane components, and finally a linear slow increase due to the hydrolysis of the various phosphorylated components and their rephosphorylation. No [^{14}C]pyruvate formation is observed if EI is omitted from the reaction mixture, indicating that phosphorylation of PTS components in the membrane (i.e., enzyme II) is responsible for the pyruvate production. The membrane fragments used in this experiment should contain the glucose-specific constitutive EII^{Man} and the inducible EII^{Mtl} since the cells were grown on mannitol. EII^{Man} can phosphorylate glucose. EII^{Mtl} phosphorylates mannitol and, to a small extent, glucitol (Jacobson et al., 1983). The glucose-specific EII and the mannitol-specific EII species do not phosphorylate each other's substrates. When mannitol is added to the complete reaction mixture at $t = 3$ min (□), there is a very rapid [^{14}C]pyruvate production. The curve levels off because all of the [^{14}C]PEP has been converted to [^{14}C]pyruvate by the excess mannitol. When glucose was added instead of mannitol (×), a somewhat slower [^{14}C]pyruvate production occurred but the [^{14}C]PEP was still exhausted within 5 min. These data confirm the presence of both EII^{Mtl} and glucose-specific EII in the membrane preparation.

Identification of 116 000-Dalton Membrane Component. The 116 000-dalton component, Figure 1C, is phosphorylated by HPr and EI. If it is an EII species, information can be obtained about its identity by determining its sugar specificity (i.e., which sugar catalyzes its dephosphorylation). The data are presented in Figure 3. In the experiment described in Figure 3A the membrane preparation was phosphorylated

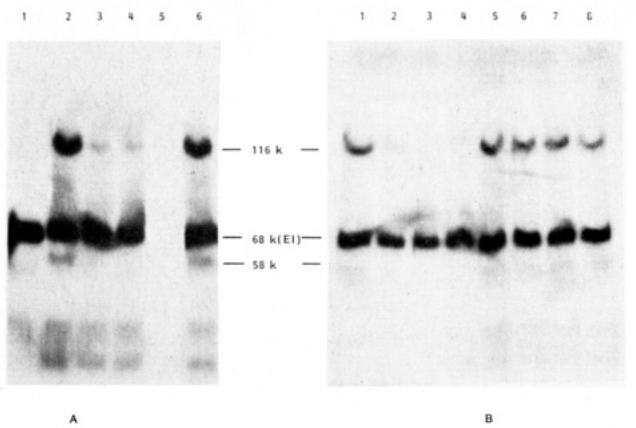

FIGURE 3: SDS gel electrophoresis and autoradiography of ^{32}P-labeled cytoplasmic membranes and effects of mannitol and glucose on phosphorylation states of enzymes. The experiments were performed at 30 °C in a phosphorylation buffer that had the same composition as described in the legend to Figure 2. (A) At $t = 0$ [^{32}P]PEP (12.5 μM) was added to three different samples that, in addition, contained exactly the same components as samples a–c described in the legend to Figure 2. At the times indicated in Figure 2, samples were withdrawn and an equal amount of solubilization buffer was added. Solubilization and electrophoresis were performed as described under Experimental Procedures. The lane numbers of the gel correspond to the sample numbers in Figure 2. (B) At $t = 0$ [^{32}P]PEP (12.5 μM) was added to cytoplasmic membranes (0.4 mg/mL) in phosphorylation buffer. The mixture was split into three portions, A–C. The following additions were made: (A) no extra additions and samples were withdrawn at $t = 2.5$ and 5.5 min (lanes 1 and 8, respectively); (B) 47 μM mannitol was added at $t = 3$ min and samples were withdrawn at $t = 3.7$, 4.3, and 5.0 min (lanes 2, 3, and 4, respectively); (C) 47 μM glucose was added at $t = 3$ min and samples were withdrawn at $t = 3.7$, 4.3, and 5.0 min (lanes 5, 6, and 7, respectively). Immediately after withdrawal the samples were diluted with an equal volume of solubilization buffer. Solubilization and electrophoresis were performed as described under Experimental Procedures.

exactly as in Figure 2 but [^{32}P]PEP in place of [^{14}C]PEP was used. At the six times indicated in Figure 2, samples were withdrawn and mixed with SDS solubilization buffer, which terminated the reaction (see legend to Figure 3). After denaturation the samples were electrophoresed on SDS-containing gels. The sample numbers in Figure 2 correspond to the lane numbers in Figure 3A. The 116 000-dalton component is visible in sample 2, taken at $t = 2.5$ min. Immediately after the addition of mannitol at $t = 3$ min there is a significant decrease in the degree of phosphorylation of the 116 000- and 58 000-dalton components but no decrease in P-EI and P-HPr (lanes 3 and 4). At $t = 9$ min, 6 min after the addition of mannitol, no phosphorylated enzyme components remain (lane 5). In the control experiment where mannitol is not added, all phosphorylated bands are still visible at $t = 9.5$ min (lane 6). Therefore, mannitol not only causes the conversion of PEP to pyruvate (Figure 2) but also selectively causes the dephosphorylation of the 116 000- and 58 000-dalton membrane components and, eventually, the dephosphorylation of P-EI and P-HPr when all PEP has been exhausted.

An identical experiment using glucose in place of mannitol (Figure 3B) shows that glucose is not capable of dephosphorylating the 58 000- and 116 000-dalton components. These data strongly support the conclusion that the 116 000- and 58 000-dalton components are mannitol-specific enzymes.

The mannitol-specific PTS described by Saier and coworkers involves only EI, HPr, and one membrane component, EII^{Mtl}, which is presumably the 58 000-dalton component observed in Figures 1C and 3. Certain published data suggest the occurrence of functional EII^{Mtl} dimer or oligomer (Leonard & Saier, 1983; Roossien & Robillard, 1984; Roossien et al., 1984). If the 116 000-dalton component is a dimer, it is extremely stable since it does not dissociate during denaturation prior to SDS gel electrophoresis. Even a 20-min incubation in 5% SDS at 60 °C did not change the ratio of dimer to monomer (not shown). During purification EII^{Mtl} is extracted from the membrane with deoxycholate, is further purified in solutions containing Lubrol PX, and ends up as a pure enzyme of 58 000 daltons showing no tendency to form 116 000-dalton aggregates on SDS gels (see Figure 1A,B). We have examined the effect of these detergents on the 116 000-dalton band in our membrane preparation. In the experiment presented in Figure 4 membranes were phosphorylated with HPr, EI, and [^{32}P]PEP. Solubilization buffer containing 2% SDS and mercaptoethanol was added. The sample was then split into several portions, and deoxycholate, NaCl, and Lubrol PX or mixtures of these reagents were added. Incubation with 0.45% DOC (lane 2) had no influence on the ratio of the 116 000- and 58 000-dalton bands. If 0.2 M NaCl was included with the 0.45% DOC, there is a slight decrease in the intensity of the 116 000-dalton band in the autoradiogram. Incubation with 0.9% Lubrol PX (lane 4) causes the 116 000-dalton band in the autoradiogram to disappear while the intensity of the 58 000-dalton band increases.

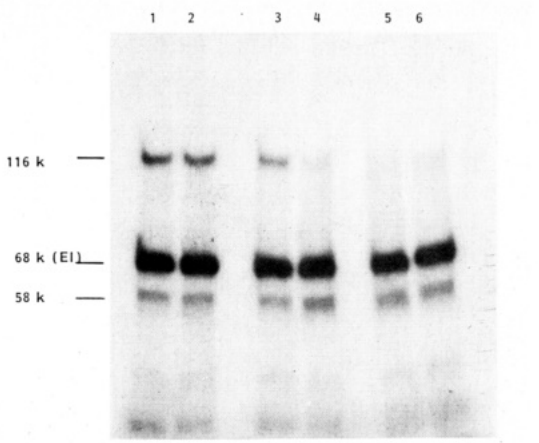

FIGURE 4: Influence of DOC and Lubrol on monomer–dimer distribution of membrane-bound EII^{Mtl}. Membranes (0.4 mg/mL) were phosphorylated at 30 °C in the same buffer as described in the legend of Figure 2. At $t = 0$ min [^{32}P]PEP was added to a final concentration of 12.5 μM. After 5 min an equal volume of solubilization buffer was added to the phosphorylated membranes. This mixture was split into six equal portions to which the following additions were made: (1) no extra additions; (2) 0.45% DOC; (3) 0.45% DOC and 0.18 M NaCl; (4) 0.9% Lubrol PX; (5) 1.8% Lubrol PX; (6) 0.9% Lubrol PX and 0.45% DOC. After 30-min incubation at 30 °C the samples were electrophoresed as described under Experimental Procedures.

Discussion

Both the EII^{Mtl}- and EII^{Glc}-catalyzed PEP-dependent sugar phosphorylation reactions follow bi-bi ping-pong kinetics, indicating that the reactions proceed via a phosphorylated EII intermediate (Roossien et al., 1984; Misset et al., 1983). The occurrence of these phosphorylated intermediates has been confirmed by pyruvate burst, pH drop, and ^{32}P-labeling experiments (Misset et al., 1983; Roossien et al., 1984; Peri et al., 1984; Raphaeli & Saier, 1980) and, in the case of EII^{Glc}, by studies on the stereochemistry of the phosphoryl group transfer reaction (Begley et al., 1982). A direct demonstration of the phosphorylated EII^{Mtl} intermediate is presented in Figure 1. ^{32}P-Labeled bands of P-EI, P-HPr, and P-EII^{Mtl} are visible in the autoradiogram after electrophoresis of a mixture consisting of [^{32}P]PEP, EI, HPr, and purified EII^{Mtl}. Both the phosphorylated and nonphosphorylated EII^{Mtl}'s migrate as a monomer.

The autoradiogram after electrophoresis of the phosphorylated membranes showed membrane-associated bands at 58 000 and 116 000 daltons. The addition of mannitol to the phosphorylated reaction mixture causes an immediate reduction in the degree of label incorporated in the 116 000- and 58 000-dalton bands in Figure 3, leading to the conclusion that these bands represent EII^{Mtl}. At the same time however, ^{32}P label is retained in the EI and HPr bands. This suggests that, under our experimental conditions, EII^{Mtl} is rapidly dephosphorylated by mannitol and only slowly rephosphorylated by P-HPr. This is consistent with the data presented in Figure 2. The rate at which the membranes are phosphorylated (open circles, Figure 2) upon addition of [^{14}C]PEP is much slower than the phosphorylation of EI and HPr (closed circles), where, in fact, a burst in [^{14}C]pyruvate is observed. The slow phosphorylation of EII^{Mtl} is due to the fact that the P-HPr concentration is far below its K_m for the membrane-bound EII^{Mtl}. At saturating concentrations of mannitol the $K_m^{P\text{-}HPr}$ for membrane-bound EII^{Mtl} is 42 μM; the K_m^{Mtl} at saturating P-HPr concentrations is 210 μM (unpublished results). Consequently, phosphoryl group transfer from P-HPr to EII^{Mtl} is rate limiting in the experiments described in Figures 2 and 3. Upon purification of EII^{Mtl} the values of these affinity constants change rather strongly. Recently we found that for the reaction catalyzed by purified EII^{Mtl}, suspended in detergent micelles, $K_m^{P\text{-}HPr}$ is 1 μM and K_m^{Mtl} is 2 μM (Roossien et al., 1984).

The autoradiogram after SDS electrophoresis of phosphorylated membrane fragments yielded only membrane-associated bands at 58 000 and 116 000 daltons, which were identified as EII^{Mtl} monomers and dimers, respectively. On the basis of its molecular weight (twice that of the monomer) we feel rather sure that the 116 000-dalton band represents an EII^{Mtl} homodimer and not a heterodimer, although this latter possibility cannot be absolutely excluded. No other membrane-associated bands that might correspond with phosphorylated glucose-specific EII could be detected in substantial amounts, although, as shown in Figure 2, glucose-specific EII is present in these membranes. The absence of ^{32}P-labeled glucose-specific EII can be explained in two ways: (i) The glucose-specific EII concentration in the membranes is much lower than the EII^{Mtl} concentration so that the bands are too weak to be detected on the autoradiogram. (ii) Glucose-specific P-EII is originally present in the phosphorylated membranes, but it is very sensitive to hydrolysis so that, in the course of solubilization or electrophoresis, the phosphoryl group dissociates.

We did not observe any detectable differences between the protein patterns of phosphorylated or nonphosphorylated membranes after SDS electrophoresis; both patterns showed a clear band at 116 000 daltons (unpublished results). From this we conclude that EII^{Mtl} is extracted as a dimer from both the phosphorylated and nonphosphorylated membranes. Incubation of the membranes with the nonionic detergent Lubrol PX transforms the P-EII^{Mtl} dimers into monomers (Figure 4). There are, apparently, very hydrophobic regions on EII^{Mtl} that constitute the interface between the subunits in the dimer. Lubrol seems to be able to occupy these sites and reduce the tendency to form dimers. Since Lubrol is present in the pure EII^{Mtl} preparations, it is not surprising that the dimers have not been observed upon electrophoresis of purified EII^{Mtl} or P-EI^{Mtl}.

In a recent study Peri et al. (1984) assigned P-EII^{Glc} to a 48 000-dalton band by comparing membranes of strains from wild-type *E. coli* with those lacking EII^{Glc} or incapable of phosphorylating it. Although their autoradiograms indicated phosphoprotein in the region of 100 000 daltons and higher, the data do not permit any conclusion about the possible presence of EII^{Glc} or EII^{Man} dimers.

Kinetic studies indicate that the dimerization of EII^{Mtl} is essential for its functioning (Leonard & Saier, 1983; Saier, 1980; Roossien et al., 1984). The fact that EII^{Mtl} can be extracted as a dimer from the membrane supports this suggestion.

Registry No. PTS, 56941-29-8; EII^{Mtl}, 37278-09-4; Mtl, 69-65-8.

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