Enzyme II of the Escherichia coli phosphoenolpyruvate-dependent phosphotransferase system: Protein-protein and protein-phospholipid interactions

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rotating carrier mechanism.

This model is based on the generally accepted but not rigorously proven assumption that high-affinity, Na^+-dependent phlorizin binding sites are localized in the same polypeptide that mediates Na^+/glucose symport. Previous attempts at solubilization and reconstitution of this transport system have failed to recover comparable activities of both Na^+-dependent sugar uptake and Na^+-dependent phlorizin binding in the same preparation (Koepsell et al., 1983). These MAb's will provide useful probes to investigate the possible role of homologous and heterologous subunit interactions in the function of this system.

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Registry No. Na, 7440-23-5; glucose, 50-99-7.

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Enzyme II of the *Escherichia coli* Phosphoenolpyruvate-Dependent Phosphotransferase System: Protein–Protein and Protein–Phospholipid Interactions†

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ABSTRACT: The mannitol-specific enzyme II (EII), purified free of phospholipid, exhibits a concentration dependence in its specific activity with P-HPr and mannitol as the donor and acceptor substrates, respectively. This concentration dependence, previously observed only in the case of the mannitol ↔ mannitol phosphate exchange reaction, indicates that an oligomeric form of the enzyme is responsible for catalyzing the phosphorylation reaction (P-HPr + mannitol → mannitol-P + HPr) as well as the exchange reaction. Kinetic analysis revealed that the monomeric enzyme has a much lower specific activity than the associated species. The specific activity can be increased by raising the steady-state level of phosphorylation of EII and also by adding phospholipid, demonstrating that phosphorylation and the binding of phospholipid facilitate the association process. Kinetic measurements and fluorescence energy transfer measurements demonstrate a strong preference of EII for phospholipids with specific head group and fatty acid composition.

Enzymes II (EII) of the bacterial phosphoenolpyruvate-dependent phosphotransferase systems are responsible for phosphorylating and transporting their specific sugar substrates across the cytoplasmic membrane. Four species of this enzyme have been purified from different microorganisms, *Escherichia coli* EII^{Mtl} (Jacobson et al., 1979, 1983a), *Staphylococcus aureus* EII^{Lac} (Schafer et al., 1981), *Salmonella typhimurium* EII^{Glc} (Erni et al., 1982), and *E. coli* EII^{Man} (Erni & Zanolari, 1985).

Two of these EII species have been observed to occur as oligomers. Erni et al. (1982) suggested that purified EII^{Glc} occurred as a dimer or trimer. The enzyme electrophoresed

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on SDS[1]–polyacrylamide gels with an apparent molecular mass of 40 000 but chromatographed on an Ultragel AcA34 column in octyl-POE at 105 000. A molecular mass of 105 000 and a sedimentation coefficient of 3.8 S were also reported as preliminary analytical ultracentrifuge data. In our laboratory EII^{Mtl}, extracted from the membrane with SDS-containing buffers, electrophoresed on SDS–polyacrylamide gels at positions corresponding to 58 000 and 116 000 daltons (Roossien & Robillard, 1984b). Patterns of [^{14}C]NEM labeling and protection against labeling by oxidants indicated that the enzyme occurred as a dimer with an oxidizable thiol contributed by each subunit (Roossien & Robillard, 1984a). We have cross-linked the subunits in the purified enzyme through these thiol groups using bifunctional maleimides and sulfhydryl oxidizing agents (Roossien et al., 1986).

The aggregation state of EII is an important issue since transporters could function by establishing solute-selective channels between the subunit interfaces. Unfortunately, the simple observation of an oligomeric species, especially in the case of intrinsic membrane proteins suspended in detergent micelles, neither confirms nor defines the functional significance of such complexes. This must be established by kinetic experiments. If the equilibrium for the association reaction does not lie too far toward one side, kinetics as a function of EII concentration can help in determining whether the monomer or oligomer is the functionally significant species.

Saier (1980) and Leonard and Saier (1983) reported different EII concentration dependencies for the phosphorylation reaction

$$\text{P-HPr} + \text{EII} \rightarrow \text{P-EII} + \text{HPr} \quad (1a)$$

$$\text{P-EII} + \text{Mtl} \rightarrow \text{EII} + \text{Mtl-P} \quad (1b)$$

vs. the exchange reaction

$$\text{Mtl-P} + \text{EII} \rightarrow \text{P-EII} + \text{Mtl} \quad (2a)$$

$$*\text{Mtl} + \text{P-EII} \rightarrow *\text{Mtl-P} + \text{EII} \quad (2b)$$

The phosphorylation rate was linearly dependent on EII concentration whereas the exchange rate showed a progressive dependence. We have made similar observations using EII^{Mtl} isolated by the same procedure (Roossien et al., 1984). One explanation of these differences is that the monomer catalyzes the phosphorylation reaction while the oligomer catalyzes the exchange reaction (Saier, 1980). Another possibility, however, is that oligomer catalyzes both reactions and the different concentration dependencies arise from different monomer/oligomer equilibrium constants for EII vs. P-EII. EII is the predominant species under the conditions used to measure steady-state exchange kinetics, but P-EII is the predominant species under the conditions used to measure steady-state phosphorylation kinetics.

This paper treats EII^{Mtl} purified with a new detergent and a simplified procedure. It is free of phospholipid, has a very high specific activity, and has a K_m for HPr very near to that observed for membrane-bound EII. The enzyme dissociates at low concentrations in the absence of phospholipid, yielding a nonlinear concentration dependence in the kinetics of the phosphorylation reaction. These results suggest that the oligomeric form is the functional form of EII^{Mtl} for catalyzing both sugar phosphorylation and transport in the membrane.

Materials and Methods

Growth of Cells and Preparation of Membrane Fragments. *E. coli* ML 308-225 were grown to A_{660} = 1.0–1.5 in medium 63 (Saier et al., 1976) containing 0.1% yeast extract and 0.5% mannitol. The procedure followed for cell harvesting, membrane preparation, and EII extraction was essentially that of Jacobson et al. (1983a). The culture medium was cooled to 5 °C by addition of ice, and from this point on, the temperature during all preparation and purification procedures was maintained at 5 °C unless otherwise specified. The chilled medium was brought to 1 mM PMSF just before harvesting, and the cells were collected in a Sharples centrifuge. The pellet was resuspended in medium 63 containing 1 mM PMSF and 1 mM sodium azide and centrifuged at 6000*g* for 10 min. The cell pellet was frozen at −20 °C until use.

Frozen cells were thawed and suspended in 20 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM sodium azide, and 1 mM PMSF (1 g of cell pellet/10 mL) and passed through a French press at 8000 psi. The suspension was centrifuged at 3000*g* for 10 min. The supernatant was then centrifuged at 200000*g* for 45 min. The supernatant was discarded, and the pellet was resuspended in the same buffer as above but at pH 8.4, with a glass potter homogenizer, and recentrifuged. The pellet was suspended in the pH 8.4 buffer (1 mL/g of starting cell wet weight). This was the membrane fragment preparation used in the isolation. It was stored in liquid nitrogen if not used immediately.

Purification of EII^{Mtl}. The following purification procedure starts with membrane fragments from 30 g of cells, wet weight. If more material is used, resin and buffer volumes must be scaled up to achieve the same level of purification. Membrane fragment suspension (30 mL) was added, dropwise over a period of 5 min, to 240 mL of extraction buffer at 25 °C with continuous stirring. The extraction buffer consisted of 20 mM Tris-HCl, pH 8.4, 0.2 M NaCl, 1 mM sodium azide, 1 mM DTT, 1 mM PMSF, and 5% deoxycholate. The suspension was incubated for 30 min at 25 °C and then cooled on ice and centrifuged at 140000*g* for 45 min. The supernatant was loaded (80 mL/h) on a hexylagarose column (20 × 2.5 cm) preequilibrated with 250 mL of extraction buffer. The column was washed with 270 mL of the same buffer and eluted with a 500-mL linear gradient of 0–2.0% Lubrol PX in the same buffer lacking PMSF. The wash and elution steps were done at a rate of 80 mL/h. The fractions were examined for mannitol phosphorylation activity following the procedure given below. The peak fraction and fractions on both sides of the peak with activities of 50% or more of the peak fraction activity were pooled. Care was taken not to include in the pool a shoulder that sometimes was found in front of the peak. EII eluted in the gradient at approximately 310 mL. The pool was dialyzed for 24 h against 1 L of 20 mM Tris-HCl, pH 8.4, 1 mM azide, and 1 mM DTT containing 0.35% decylpoly(ethylene glycol) 300 (decyl-PEG) and 0.35% deoxycholate. It was then dialyzed for 24 h against 1 L of the same buffer lacking deoxycholate. The dialyzed pool was diluted 3 times with the same buffer and loaded (30 mL/h) on a butylagarose column (30 × 1.5 cm) preequilibrated with the same buffer. The column was washed with 60 mL of the buffer and eluted with a 200-mL linear gradient of 0–200 mM NaCl in the buffer. The elution speed was 15 mL/h. EII eluted at 90–95 mL. The peak fraction and fractions with EII activity 50% or higher of the peak fraction activity were pooled and frozen in liquid nitrogen. The recovery was 25% of the starting activity.

[1] Abbreviations: PTS, phosphoenolpyruvate-dependent phosphotransferase system; decyl-PEG, decylpoly(ethylene glycol) 300; PEP, phosphoenolpyruvate; DMPC, dimyristoylphosphatidylcholine; DMPG, dimyristoylphosphatidylglycerol; DOPC, dioleoylphosphatidylcholine; DOPG, dioleoylphosphatidylglycerol; PPPC, 1-palmitoyl-2-(pyrenyldecanoyl)-*sn*-glycero-3-phosphocholine; SDS, sodium dodecyl sulfate; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; NEM, *N*-ethylmaleimide.

If the same resin was used for more than three isolations, the level of purification, as judged by SDS gel electrophoresis, decreased. The resins were washed after each purification with 2 300-mL volumes of 1 M NaCl and then 4 volumes of water followed by 2 200-mL volumes of 2.5% deoxycholate with a 0.5-h incubation period during each wash. The resin was rinsed with water until all of the deoxycholate had been removed. It was stored in 0.5 M NaCl–1 mM azide in the refrigerator. Before the following purification, 1 mM penicillin was added, and the resin was incubated at room temperature overnight.

SDS–polyacrylamide gel electrophoresis was performed on 10% polyacrylamide slab gels containing 0.1% SDS essentially as described by Laemmli (1970). The electrophoresis buffer, pH 8.4, contained 6.32 g/L Tris, 3.94 g/L glycine, and 0.1% SDS. The protein was denatured by 1:1 dilution with 0.125 M Tris-HCl, pH 8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, and 0.02% bromophenol blue and incubation for 30 min at 30 °C. This solution was then loaded on gels. The silver staining was done by the method of Wray et al. (1981).

Phosphate determination was done according to the microphosphate procedure of Chen et al. (1956). EII was dialyzed against 20 mM Tris-HCl, pH 8.4, 85 mM NaCl, 1 mM DTT, 1 mM azide, and 0.35% decyl-PEG. The blank was the same solution without enzyme. The procedure was calibrated with a sodium phosphate solution and yielded a calibration curve of 0.012 A_{820}/nmol of P_i. EII was concentrated to 4.4 μM on Centricon-30 concentrators (Amicon). No inorganic phosphate could be detected with up to 9 nmol of EII. Assuming a 1:1 molar ratio of phospholipid to EII, 9 nmol of EII should have resulted in an absorbance increase of 0.11.

Determination of EII Concentration by the Pyruvate Burst Method. This procedure separates [^{14}C]pyruvate that is formed from [^{14}C]PEP when the PTS components become phosphorylated. Columns containing 1 mL of Dowex AG 1-X2 (200–400 mesh) were prepared in Pasteur pipets. Care was taken to use columns with the same flow rate. They were equilibrated with 3 1.5-mL volumes of 0.2 M HCl followed by 3 volumes of water. The columns were placed over scintillation vials. When samples were loaded on the column, the column was first filled with water and the sample pipetted directly into the water. This volume and 4 1.5-mL volumes of 0.125 M NaCl were collected in the same vial (the [^{14}C]-pyruvate fraction). The columns were then placed over a second set of vials and washed with 4 1.5-mL volumes of 1 M NaCl to elute the [^{14}C]PEP. Packard emulsifier scintillator 299 was added, and the vials were counted in a Nuclear Chicago Mark II scintillation counter. The burst was carried out in 50 mM sodium phosphate, pH 6.5, 1 mM magnesium chloride, 2 mM NaF, 4 mM DTT, 0.2 μM EI, 1 μM HPr, and 0.2% decyl-PEG. The reaction mixtures (200 μL) containing these components plus 0, 1, 2, and 3 volumes of EII were warmed at 30 °C for 10 min, after which a 5–6-fold excess of [^{14}C]PEP over PTS proteins was added. Samples of 30 μL were removed every 4 min for 30 min and pipetted directly onto the columns described above. The pyruvate concentrations were extrapolated to $T = 0$ to determine the burst value. The EII concentration was determined by the difference in the burst values for the reaction mixtures containing 1, 2, and 3 volumes of EII.

Determination of Mannitol Phosphorylation Activity. Mannitol phosphorylation activity was measured in reaction mixtures of 100–200-μL volumes containing 25 mM Tris-HCl, pH 7.6, 10 mM NaF, 5 mM $MgCl_2$, 10 mM K-PEP, 0.35% decyl-PEG, 10 μM HPr, and 0.1 μM EI and EII. The reaction mixtures were prepared on ice and then preincubated at 30 °C for 5–10 min, after which [^{14}C]mannitol was added. Incubation was continued at the same temperature, and aliquots were withdrawn at given intervals and processed for [^{14}C]-Mtl-P with the following procedure. Glass tubes (10 cm × 0.5 cm i.d.) sealed on the bottom with a coarse glass fritted disc and loaded with a 0.5-mL bed volume of Dowex AG 1-X2 (50–100 mesh) were washed with 3 volumes (1.4 mL each) of 0.2 N HCl followed by 3 volumes (2.5 mL each) of water. Aliquots (30–60 μL) of the reaction mixture were pipetted onto a column just after it was filled with water. Each column was rinsed with 3 volumes (2.5 mL each) of water to remove unreacted [^{14}C]Mtl and then placed over a mini scintillation vial and eluted with 2 volumes (1.4 mL each) of 0.2 N HCl to remove the [^{14}C]Mtl-P. A total of 2 mL of Packard emulsifier scintillator 299 was added to each vial, and they were counted.

HPr and EI were isolated as described (Dooijewaard et al., 1979; Misset & Robillard, 1982).

Phospholipid stock solutions were prepared by placing aliquots of a phospholipid/chloroform stock solution in test tubes and evaporating with a stream of dry nitrogen gas. Water or a salt solution was added, and the tubes were vortexed until the phospholipid was suspended.

The phospholipids DMPC, DMPG, DOPC, and DOPG were generous gifts of Dr. J. De Gier, Utrecht. The fluorescent probe 1-palmitoyl-2-(pyrenyldecanoyl)-*sn*-glycero-3-phosphocholine (PPPC) was a generous gift of Dr. K. Wirtz, Utrecht.

[1-^{14}C]Phosphoenolpyruvate and D-[1-^{14}C]mannitol were purchased from the Radiochemical Centre, Amersham. The specific activity was checked by isotope dilution procedures.

Phosphoenolpyruvate (monopotassium salt), egg yolk phosphatidylglycerol, and DTT were obtained from Sigma. All other chemicals were of reagent grade.

RESULTS

Figure 1 presents a silver-stained SDS–polyacrylamide gel of the hexylagarose and butylagarose pools from the EII isolation procedure described above. When the gel is overloaded, faint bands can be observed in the butylagarose pool. Integration of densitometer traces shows that the intense band running at the position of EII in the butylagarose pool accounts for more than 99% of the staining intensity in the gel.

EII Self-Association Is Required for Phosphorylation Activity. A plot of the specific phosphorylation activity as a function of the EII concentration in Figure 2 (●) shows that the specific activity varies with the enzyme concentration, suggesting that EII must achieve an associated state before reaching maximum activity. This behavior is different from that reported earlier by ourselves and Saier in which only the specific exchange activity and not the specific phosphorylation activity was enzyme concentration dependent. This difference could arise from the use of decyl-PEG as a detergent in place of Lubrol PX either in the assay or in the purification procedure. These alternatives have been checked by replacing decyl-PEG with Lubrol PX in the purification procedure; 0.5% Lubrol PX was used in the dialysis step, and 0.05% Lubrol PX was employed in the butylagarose chromatography. The EII concentration dependence of the specific phosphorylation activity can still be observed even when the enzyme was purified in Lubrol PX (open symbols). However, the enzyme concentration at which maximum activity is achieved is shifted to lower values. In addition, a higher specific activity was reached at low concentrations of Lubrol PX. The maximum

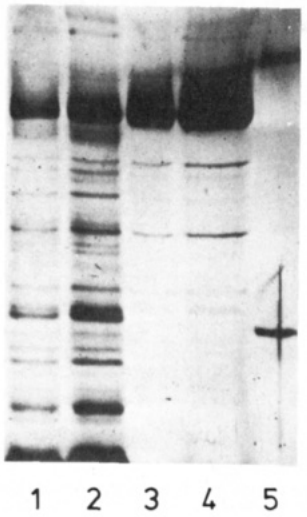

FIGURE 1: SDS–polyacrylamide gel electrophoresis of the hexylagarose and butylagarose pools of EII obtained during purification of the enzyme. A 10% cross-linked polyacrylamide gel was used. Bovine serum albumin and chymotrypsinogen were run as standards in lane 5. The vertical streak in this lane is a staining artifact. Lanes 1 and 2 are 10 and 20 μL, respectively, of the dialyzed EII pool from the hexylagarose chromatography step. Lanes 3 and 4 are 20 and 40 μL, respectively, of the EII pool from the butylagarose chromatography step. The anode is at the bottom of the gel. Procedures for preparing the protein, running the gel, and silver staining are stated under Materials and Methods.

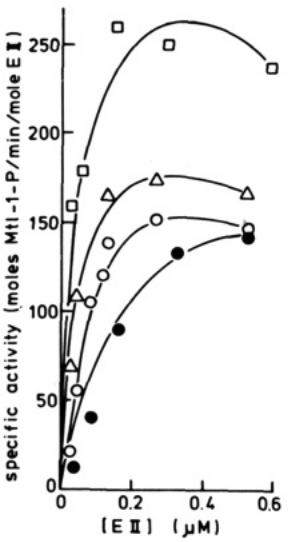

FIGURE 2: EII concentration dependence of the specific phosphorylation activity. (Closed symbols) The reaction mixture contained 25 mM Tris-HCl, pH 7.6, 10 mM NaF, 5 mM $MgCl_2$, 10 mM K-PEP, 5 mM DTT, 0.35% decyl-PEG, 10 μM HPr, 0.1 μM EI, and the stated concentrations of EII that had been purified in decyl-PEG. The samples were preincubated at 30 °C for 5–10 min, after which 3 mM [^{14}C]Mtl was added. Aliquots were taken every 4 min for 32 min and processed for Mtl-P as described under Materials and Methods. Separate measurements over a range of EI concentrations from 0.05 to 0.5 μM at fixed HPr and EII concentrations showed that 0.1 μM EI was not rate limiting. (Open symbols) Activity measurements were done under the conditions described above except that, during purification, the buffer in the dialysis step between the hexyl- and the butylagarose chromatographies contained 0.5% Lubrol PX. Decyl-PEG was never used. Furthermore, 0.05% Lubrol PX was used instead of decyl-PEG during equilibration and elution of the butylagarose column. The 0.35% decyl-PEG in the assay mixture has been replaced by the following detergents: (△) 0.4% Lubrol PX; (□) 0.04% Lubrol PX; (○) 0.4% decyl-PEG plus 0.003–0.03% Lubrol PX.

specific activities are not true V_{max} values because the activities were measured with subsaturating concentrations of HPr (see below). Higher concentrations of Lubrol PX reduce the

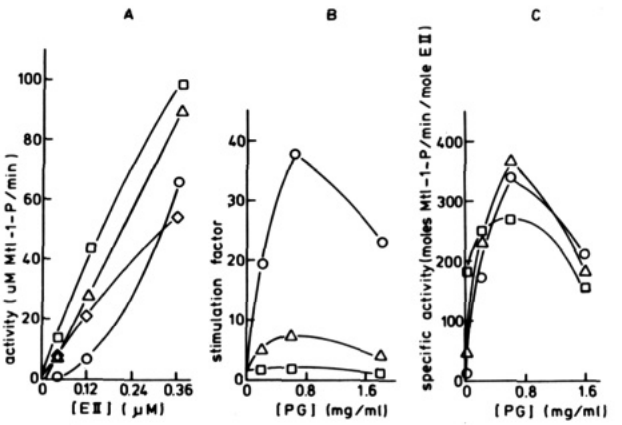

FIGURE 3: (A) Influence of phosphatidylglycerol on the EII concentration dependence of the phosphorylation activity. Reaction mixtures were prepared as described under Materials and Methods with EII that had been purified in decyl-PEG. Aliquots of a 10 mg/mL stock solution of phosphatidylglycerol in water were added to the complete reaction mixtures on ice. They were incubated at 30 °C for 30 min, after which assays were started by the addition of 3 mM [^{14}C]Mtl. Phosphatidylglycerol concentrations in the reaction mixtures were (○) 0, (△) 0.2, (□) 0.6, and (◇) 1.8 mg/mL. (B) Stimulation of phosphorylation activity as a function of the EII and phosphatidylglycerol concentration. The data are replotted from (A). The stimulation factor is the activity in the presence of PG divided by the activity in the absence of PG. The EII concentrations are (○) 0.04, (△) 0.12 and (□) 0.36 μM. (C) Specific activity of various concentrations of EII as a function of the phospholipid concentration. The data are replotted from (A). The EII concentrations are (○) 0.04, (△) 0.12, and (□) 0.36 μM.

specific activity. The enzyme purified in Lubrol PX and diluted into decyl-PEG before assaying (○) possesses the same specific activity as the enzyme that was both purified and assayed in decyl-PEG (●).

Phospholipids Facilitate Self-Association of EII. The data in Figure 2 suggest that protein–protein and protein–detergent interactions are critical for the activity of EII. Figure 3 considers the phospholipid dependence of the specific activity. Inorganic phosphate analysis of the purified enzyme used in this study showed less than 1 mol of inorganic phosphate/mol of enzyme (cf. Materials and Methods). Addition of egg yolk phosphatidylglycerol to the enzyme had a marked effect on the enzyme concentration dependence of the activity (Figure 3A). The progressive dependence on EII concentration seen in the absence of added phospholipid was removed by addition of 0.2–0.6 mg of phosphatidylglycerol, indicating that phospholipid stimulates the association process and that, in approximately 0.4–0.6 mg/mL phosphatidylglycerol, EII is completely associated. Higher phosphatidylglycerol concentrations resulted in lower EII activities (see below).

Figure 3B presents the ratio of the activity with vs. without phosphatidylglycerol as a function of the phosphatidylglycerol concentration at various concentrations of EII in the assay mixture. The activity was stimulated up to 37 times at 0.04 μM EII. The stimulation was lower at higher EII concentrations where a larger fraction of the enzyme already seemed to occur in the associated form. Concentrations of phosphatidylglycerol higher than approximately 0.6 mg/mL appeared to be inhibitory. This is apparently related to the structure of the EII–detergent–lipid micelle since the maximum in Figure 3B can be shifted to higher or lower phospholipid concentrations by altering the lipid detergent ratio. Increasing the amount of detergent at fixed concentrations of EII and lipid shifted the maximum to the right (cf. Figure 5). The specific activity, plotted in Figure 3C, reflects these different stimulation values but also shows an unexpected EII concen-

tration dependence. EII, at high concentrations, possessed a lower specific activity in the presence of moderate to high amounts of phospholipid. This may reflect an aggregation of EII to a species with a lower specific activity (see Discussion).

Kinetic Alterations upon Addition of Phospholipid. The changes in activity occurring upon addition of phosphatidylglycerol or upon raising the concentration of EII in the absence of phospholipid could be due to alterations in K_m or V_{max} parameters, or both. We have examined the phosphorylation kinetics in the presence or absence of 0.6 mg/mL phosphatidylglycerol at HPr concentrations ranging from 6 to 75 μM using 0.09 μM EII and 2.5 mM mannitol. The rest of the components in the reaction mixture were identical with those listed under Materials and Methods as was the procedure for preparing the reaction mixtures and following the formation of Mtl-P. The results showed that phospholipid changed the V_{max} but not the K_m. the V_{max} is 960 mol of Mtl-1-P min^{-1} (mol of EII)$^{-1}$ in the absence of phosphatidylglycerol. This value agrees well with that measured previously [1000 mol of Mtl-1-P min^{-1} (mol of EII)$^{-1}$] (Roossien et al., 1984). The V_{max} is 1800 mol of Mtl-1-P min^{-1} (mol of EII)$^{-1}$ in the presence of 0.6 mg/mL phosphatidylglycerol. The K_m for P-HPr was 24 μM. This value is close to that observed in membrane-bound preparations of EII. It is higher than that reported previously by Roossien et al. (1984) and Jacobson et al. (1983a).

These measurements and those reported in Figures 2 and 3 involved saturating concentrations of EI so that all HPr was present as P-HPr. The P-HPr concentrations were also high enough to ensure that the steady-state form of EII was predominantly P-EII. The combined data show that P-EII is dissociated at low concentrations in the absence of phospholipid.

Effect of Phosphorylation on Association State of EII. Two issues have not yet been clarified: whether the steady-state level of EII phosphorylation influences the association–dissociation equilibrium of the enzyme and whether monomeric EII is active but at a considerably lower specific activity than the associated enzyme. Both questions can be addressed by studying the HPr concentration dependence of the kinetics. The steady-state degree of phosphorylation of EII can be controlled by choosing the correct concentrations of phosphoryl donor and acceptor substrates, P-HPr and mannitol, respectively. At high concentrations of P-HPr, the rate of Mtl phosphorylation will be controlled by the rate of reaction 1b, and EII will exist as P-EII in the steady state. At low P-HPr and high Mtl concentrations, reaction 1a will be rate limiting, and EII will be dephosphorylated in the steady state. If the association–dissociation equilibrium is not effected by the degree of phosphorylation of EII and if only dimeric EII is enzymatically active, a Lineweaver–Burk plot should report a single linear relationship between $1/V$ and $1/[HPr]$ over the entire HPr concentration range. This will lead to one value of V_{max}, that of associated EII. If, on the other hand, phosphorylation shifts the equilibrium between monomeric and associated EII and the specific activity of each species is different, a Lineweaver–Burk plot will not be linear over the whole HPr concentration range. We have examined the rates of Mtl phosphorylation at low HPr concentrations ranging from 2 to 800 nM. The data in Figure 4 show that the Lineweaver–Burk plot is not linear over the entire HPr concentration range examined. There is a linear portion of the Lineweaver–Burk plot at low HPr, between 2.5 and 14 nM. The V_{max} extrapolated from this portion of the plot was 3 mol of Mtl-1-P min^{-1} (mol of EII)$^{-1}$. Above 15 nM HPr, the

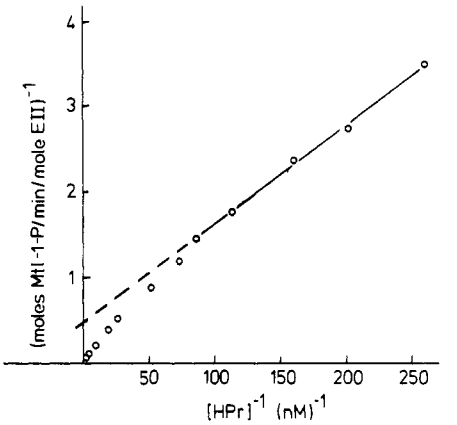

FIGURE 4: Lineweaver–Burk plot of the rate of PEP-dependent mannitol phosphorylation as a function of the HPr concentration. All measurements were made under the same conditions of mannitol, EI, and EII concentration. The reaction conditions were 25 mM Tris-HCl, pH 7.6, 10 mM NaF, 5 mM $MgCl_2$, 0.35% D-PEG, 10 mM K-PEP, 0.5 μM EI, and 0.16 μM EII. The reaction mixtures were prepared on ice and then preincubated at 30 °C for 10 min, and the reactions were initiated by the addition of [^{14}C]mannitol to a final concentration of 180 μM (sp act. 55 mCi/mmol). Samples were withdrawn at various time intervals and processed as stated under Materials and Methods.

Lineweaver–Burk plot curved downward due to rates higher than expected from the extrapolated V_{max}. When measured at HPr concentrations between 1 and 50 mM and at saturating mannitol concentrations, a V_{max} of approximately 1000 mol of Mtl-1-P min^{-1} (mol of EII)$^{-1}$ was obtained. This is the same value as that published in previous reports using enzyme purified by different procedures (Roossien et al., 1984; Jacobson et al., 1983).

These data show that phosphorylation alters the association–dissociation equilibrium of purified EII in detergent; P-EII associates more readily than EII. In addition, they show that the monomeric species is enzymatically active albeit at a rate negligible by comparison with that of the associated species.

Phospholipid Specificity. Even though egg yolk phosphatidylglycerol stimulated EII 2.5-fold, *E. coli* phosphatidylglycerol stimulated no more than a factor of 1.1 (Jacobson et al., 1983a,b). These observations suggest that the fatty acid composition may be as essential as the head group composition, if not more so, in the activation process. This is examined in Figure 5 with four pure phospholipids. In the absence of phospholipid, 0.04 μM EII had a specific phosphorylation activity of 10 mol of Mtl-1-P min^{-1} (mol of EII)$^{-1}$. DOPG produced virtually no stimulation and DOPC stimulated only poorly when compared with the stimulation produced by egg yolk phosphatidylglycerol (Figure 5, left panel). DMPC was considerably more effective than DMPG (Figure 5, right panel); however within the glycerol family, DMPG was more effective than DOPG. Thus, the nature of both the head group and the fatty acid chain is important in generating a highly active EII species. Myristoyl is much more effective than the oleoyl hydrocarbon chain, and choline is more effective than the glycerol moiety. The filled diamonds in the right-hand panel show the stimulation observed by DMPC in 0.7% decyl-PEG. Comparison of these data with the open diamonds (0.35% decyl-PEG) shows that maximum activity is dependent on an optimal detergent:lipid ratio. This explains the decreased activity in Figure 3B,C at high phosphatidylglycerol concentrations.

EII–Phospholipid Interactions Demonstrated by Fluorescence Energy Transfer. EII possesses four tryptophan residues (Lee & Saier, 1983). The fluorescence from these residues

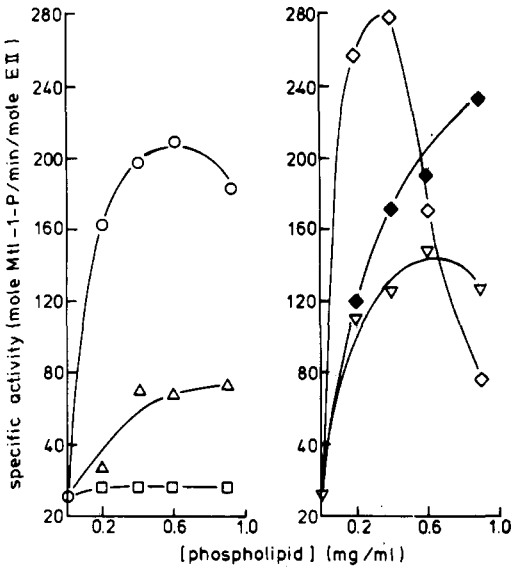

FIGURE 5: Influence of the phospholipid composition on the specific phosphorylation activity. Stock solutions (2 mg/mL) of DMPC, DOPC, DOPG, and egg yolk phosphatidylglycerol were prepared in water at the desired concentrations as described under Materials and Methods. Because of solubility problems, a 4 mg/mL stock solution of DMPG was prepared in 0.8% decyl-PEG and 0.4 M NaCl. After the reaction mixture consisting of the components listed in the legend to Figure 2 and 0.04 μM EII was prepared, the phospholipid stock solution was added, and the mixtures were preincubated at 30 °C for 30 min. [^{14}C]Mtl was added, and the activity was followed as described under Materials and Methods. In the case of the DMPG measurements, the final decyl-PEG concentration was maintained at 0.35% by use of a reduced detergent concentration in the initial reaction mixture. The final NaCl concentration in this reaction mixture was 90 mM. Control experiments showed that, in the absence of phospholipid, this concentration of NaCl resulted in a 20% lower EII activity relative to samples lacking NaCl. The uncorrected values are given in the figure. The phospholipids used were (○) egg yolk phosphatidylglycerol, (△) DOPC, (□) DOPG, (◇), DMPC, and (▽) DMPG. (Filled symbols) Effect of changing the detergent concentration on DMPC stimulation. All components in the reaction mixture are the same as (◇) excpet that decyl-PEG is present at 0.70% instead of 0.35%.

can be used to report on their environment and conformational changes resulting from interaction of EII with other components of the PTS. The experiments reported in Figure 6 employ fluorescence energy transfer to examine the interaction between EII and two of the phospholipids studied in Figure 5. Panel 1, Figure 6, shows the fluorescence emission spectrum of EII. The spectrum (a) shows an emission maximum at 330 nm, which is characteristic for tryptophan residues in a hydrophobic environment. Addition of 4 equiv of the fluorescent phospholipid probe 1-palmitoyl-2-(pyrenyldecanoyl)-*sn*-glycero-3-phosphocholine (PPPC) resulted in a sizable decrease in the intensity of the tryptophan emission peak and an enhanced emission at approximately 377 and 395 nm (spectrum b). Spectrum c shows the fluorescence spectrum of a similar concentration of PPPC in buffer without EII. It is clear from a comparison of these spectra that the decrease in the tryptophan emission intensity at 330 nm and the new intensity at 377 and 395 nm in panel 1 are due to resonance energy transfer between the tryptophan residues in the protein and the phospholipid probe, indicating that the phospholipid probe binds to EII.

DMPC strongly stimulated the EII activity in Figure 5. The effect of DMPC on the interaction between EII and PPPC is examined in panel 2 of Figure 6. Addition of DMPC (final concentration, 40 μg/mL) to a solution of EII and PPPC resulted in an enhanced pyrene emission spectrum with little or no change in the amount of tryptophan quenching. The concentration of DMPC yielding this effect was a factor of 10 lower than the concentration required for maximum stimulation of EII activity in Figure 5. Panel 3 shows a similar experiment with DOPG; this phospholipid was ineffective in stimulating EII activity in Figure 5. DOPG at concentrations ranging from 20 to 160 μg/mL had no influence on the level of quenching of the tryptophan emission peak or the enhanced emission of the pyrene bands. At 320 μg/mL, a slight additional quenching of the tryptophan fluorescence and stimulation of the pyrene emission bands occurred. However, subsequent addition of DMPC to a final concentration of only 40 μg/mL resulted in enhanced pyrene emission without additional quenching of the tryptophan fluorescence. The level of enhancement was the same as in panel 2 after correcting for the difference of a factor of 2 in the PPPC concentration in the two experiments.

Discussion

Specific EII–Phospholipid Interactions. The alteration of EII–PPPC energy-transfer parameters by individual phospholipids is evidence for binding to selective sites on the enzyme. Since the enzyme contains four tryptophans, we cannot yet specify whether the quenching involves a single tryptophan or more than one. The different response of the stimulated PPPC emission upon addition of low DMPC concentrations vs. high concentrations of DOPG mirrors the different ability of these two phospholipids to stimulate EII activity. DOPG has very little effect on EII activity and alters the PPPC-stimulated emission only at high concentrations while DMPC strongly effects EII activity and alters PPPC-stimulated emission very effectively at low concentrations even in the presence of DOPG. This suggests either different binding sites for DMPC and DOPG or a much stronger binding of DMPC over DOPG to the same site. The stimulated emission can be explained in several ways. Binding of the phospholipid could alter the conformation of the enzyme resulting in (i) increased tryptophan absorption, (ii) increased quantum yield of tryptophan fluorescence, and (iii) a decrease in static quenching of the bound PPPC. Binding could also displace PPPC from certain sites not contributing to the energy-transfer process and encourage binding at other sites with higher energy-transfer efficiencies.

Effect of Phospholipid. Phospholipid fulfills two functions in the reconstituted system. It stimulates EII self-association as shown by the change in the EII concentration dependence of the activity in Figure 3A. It also stimulates the specific phosphorylation activity of the associated enzyme as is evident from the different effects of the pure phospholipids in Figure 5. Two unexplained observations are (1) decreased specific activity at high phospholipid concentration and (2) decreased specific activity at high EII concentrations. The former may be due to phospholipid binding to EII hydrophobic interfaces, which prevents or disturbs EII self-association. It may also be due to segregation of EII monomers into separate micelles as a result of the altered detergent:lipid ratio. The later may reflect an EII aggregation to a less active form. Radiation inactivation data show that EII occurs in two associated forms in the membrane, an active, dimeric form and a less active trimeric or tetrameric form (Pas & Robillard, 1987).

An indication of a preference for specific fatty acids can be found in the reports of Jacobson that *E. coli* phosphatidylglycerol was much less effective in stimulating EII activity when compared with egg yolk phosphatidylglycerol (Jacobson et al., 1983a,b). In this current investigation, we observe an EII preference for the nature of both the polar head group and the fatty acid chain. Caution is required, however, in inter-

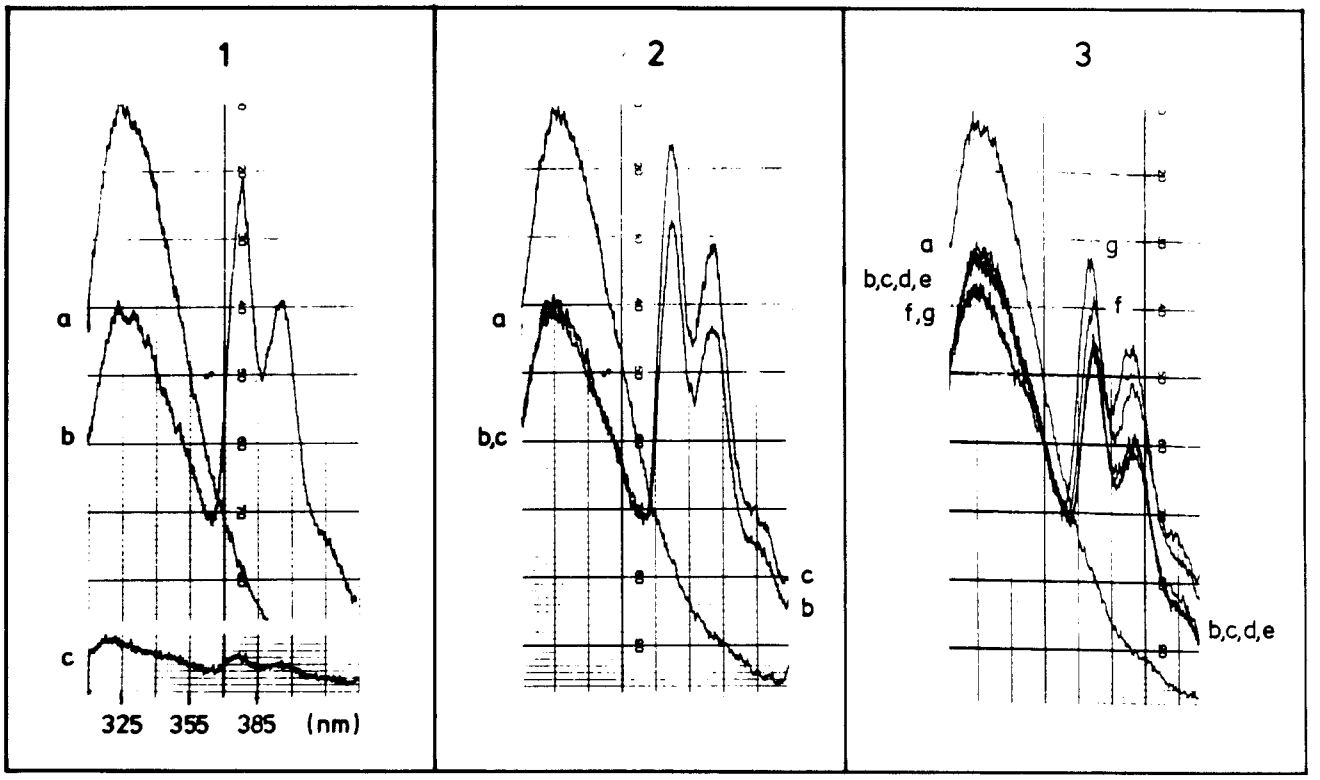

FIGURE 6: Influence of phospholipids on resonance energy transfer between EII tryptophan residues and 1-palmitoyl-2-(pyrenyldecanoyl)-*sn*-glycero-3-phosphocholine. Fluorescence measurements were carried out on a Perkin-Elmer MPF-43 spectrofluorometer with excitation at 290 nm using an excitation slit of 4 nm and an emission slit of 10 nm. EII stock solutions in 20 mM Tris-HCl buffer, pH 8.4, containing 1 mM DTT, 0.1 M NaCl, and 0.35% decyl-PEG were diluted 10 times into 5 mM HEPES buffer, pH 7.5, containing 150 mM NaCl. PPPC was added from a 0.1 mM stock solution in ethanol. DMPC and DOPG were added from 2 mg/mL stock solutions in water. Each sample was incubated in the cuvette, in the spectrophotometer at room temperature for 5 min with continuous stirring, after which the spectrum was measured. PPPC (1 μM) had no effect of EII mannitol phosphorylation activity. (Panel 1) (a) 0.26 μM EII; (b) 0.26 μM EII plus 1 μM PPPC; (c) 0.6 μM PPPC in buffer. (Panel 2) (a) 0.26 μM EII; (b) 0.26 μM EII plus 1 μM PPPC; (c) 0.26 μM EII, 1 μM PPPC, and 40 μg/mL DMPC. (Panel 3) (a) 0.26 μM EII; (b) 0.26 μM EII plus 0.5 μM PPPC; (c–f) 0.26 μM EII, 0.5 μM PPPC, and 20, 40, 80, and 320 μg/mL DOPG, respectively; (g) 0.26 μM EII, 0.5 μM PPPC, 320 μg/mL DOPG, and 40 μg/mL DMPC.

preting these results. It is well-known that detergents can alter the size and composition of mixed micelles, and they clearly influence EII activity in the phospholipids examined. Reconstitution studies in the absence of detergent are essential before making any definite conclusions concerning fatty acid and head group specificity.

EII Association. Self-association is essential for maximum phosphorylation and exchange activity. This can be achieved either by increasing the EII concentration or by the addition of phospholipid at low concentrations of EII. Previous reports by ourselves and Saier were only able to show an EII concentration dependence in the specific exchange activity but not in the specific phosphorylation activity (Rossien et al., 1984; Saier, 1980). Those earlier preparations probably contained small amounts of bound phospholipid capable of altering the equilibrium between the associated and dissociated form of the purified enzyme. Since phosphorylation tends to shift the enzyme to the associated form, the presence of phospholipid plus the high steady-state level of phosphorylation could have brought the enzyme into the fully associated form even at low enzyme concentrations and eliminated any concentration dependence of the specific phosphorylation activity. By contrast, the conditions of the exchange reaction favor the dissociated form of the enzyme. The low steady-state level of P-EII and the high concentration of Mtl-1-P (Rossien et al., 1984) both force the enzyme toward the dissociated state, which would result in an enzyme concentration dependence of the specific exchange activity.

Membrane-bound EII monomers are most likely associated due to the presence of phospholipids. Thus, the stimulation of association by phosphorylation is not physiologically significant but rather a fortuitous artifact characteristic of the purified enzyme. The nature of the associated species cannot be specified from the present data. However, several lines of evidence indicate that the functional species is a dimer both for the purified enzyme suspended in detergent micelles and for the enzyme in the membrane. Labeling of the thiols in the purified enzyme was consistent with a dimer, as was cross-linking with short cross-linkers or oxidation of the activity-linked dithiols to a disulfide (Roossien & Robillard, 1984a, 1986). Experiments with the membrane-bound enzyme showed that a dimeric species could be extracted from the membrane and visualized on SDS gels (Rossien & Robillard, 1984b; Stephan & Jacobson, 1986). Radiation inactivation studies suggest that the minimum size of the functionally active species in the membrane is a dimer (Pas & Robillard, 1987).

NEM labeling studies on purified EII detected three cysteines per peptide chain, one of which was thought to be involved in a stable disulfide between the subunits because it could only be labeled after treating the enzyme with urea and DTT (Rossien & Robillard, 1984a). However, we have not been able to detect a stable dimer on SDS–polyacrylamide gels using a purified enzyme preparation treated at 30 °C with SDS and DTT. If it existed, the stable dimer would have been detected since these are mild conditions that do not fully denature EII (Roossien & Robillard, 1984b). Current data suggest that there is no stable intermolecular disulfide. The third cysteine and a fourth one found in the sequence work of Lee and Saier (1983) are most likely buried in the hydrophobic interior and require strong denaturant and DTT to

expose them to alkylating agents. The lack of a stable intermolecular disulfide is also consistent with the EII concentration-dependent association data presented in this paper.

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Registry No. EII, 37278-09-4; DMPC, 13699-48-4; DMPG, 61361-72-6; DOPC, 10015-85-7; DOPG, 62700-69-0; PPPC, 95864-17-8; mannitol, 69-65-8.

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A Deuterium and Phosphorus-31 Nuclear Magnetic Resonance Study of the Interaction of Melittin with Dimyristoylphosphatidylcholine Bilayers and the Effects of Contaminating Phospholipase A_2

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ABSTRACT: The interaction of bee venom melittin with dimyristoylphosphatidylcholine (DMPC) selectively deuteriated in the choline head group has been studied by deuterium and phosphorus-31 nuclear magnetic resonance (NMR) spectroscopy. The action of residual phospholipase A_2 in melittin samples resulted in mixtures of DMPC and its hydrolytic products that underwent reversible transitions at temperatures between 30 and 35 °C from extended bilayers to micellar particles which gave narrow single-line deuterium and phosphorus-31 NMR spectra. Similar transitions were observed in DMPC–myristoyllysophosphatidylcholine (lysoPC)–myristic acid mixtures containing melittin but not in melittin-free mixtures, indicating that melittin is able to stabilize extended bilayers containing DMPC and its hydrolytic products in the liquid-crystalline phase. Melittin, free of phospholipase A_2 activity, and at 3–5 mol % relative to DMPC, induced reversible transitions between extended bilayers and micellar particles on passing through the liquid-crystalline to gel phase transition temperature of the lipid, effects similar to those observed in melittin–acyl chain deuteriated dipalmitoylphosphatidylcholine (DPPC) mixtures [Dufourc, E. J., Smith, I. C. P., & Dufourcq, J. (1986) *Biochemistry 25*, 6448–6455]. LysoPC at concentrations of 20 mol % or greater relative to DMPC induced transitions between extended bilayers and micellar particles with characteristics similar to those induced by melittin. It is proposed that these melittin- and lysoPC-induced transitions share similar mechanisms. The effects of melittin on the quadrupole splittings and T_1 relaxation times of head-group-deuteriated DMPC in the liquid-crystalline phase share features similar to the effects of metal ions on DPPC head groups [Akutsu, H., & Seelig, J. (1981) *Biochemistry 20*, 7366–7373], indicating that the conformational properties of the choline head group in PC bilayers may be affected by melittin and by metal ions in a similar manner.

Many studies on the interaction of bee venom melittin (Habermann & Jentsch, 1967) with model membranes composed of synthetic lipids have been carried out with the aim of defining the structural basis for the lytic effects of the peptide (Bernheimer & Rudy, 1986). It is still not clear, however, whether melittin acts by perturbing the structural organization of bilayer membrane lipids or by inducing discrete pores through which ions may diffuse, producing osmotic lysis.

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