Subunit Interactions of the *Escherichia coli* Mannitol Permease: Correlation with Enzymic Activities†

Megan M. Stephan and Gary R. Jacobson*

Department of Biology, Boston University, Boston, Massachusetts 02215

Received December 20, 1985; Revised Manuscript Received March 7, 1986

ABSTRACT: A fraction of the phosphorylated form of the *Escherichia coli* mannitol permease (enzyme II^{Mtl}) of the sugar phosphotransferase system can be extracted from the membrane in a dimeric form [Roossien, F. F., & Robillard, G. T. (1984) *Biochemistry 23*, 5682–5685]. Using *E. coli* minicells in which this protein can be specifically labeled with [^{35}S]methionine, we show in this paper that part of the unphosphorylated form of enzyme II^{Mtl} can also be extracted from the membrane as a dimer. We further demonstrate that both phosphoenolpyruvate-dependent phosphorylation of the permease and conditions promoting turnover of the enzyme decrease the amount of extractable dimer. Thus, the dimer of these forms of the enzyme appears to be less stable than that of the unmodified form, at least in detergent solution. In contrast, inorganic phosphate, which activates the permease-catalyzed phospho exchange between mannitol 1-phosphate and mannitol ("transphosphorylation"), stabilizes the dimer. These results support the hypothesis that the mannitol permease dimer is more active in transphosphorylation than the monomer. Treatment of minicell membranes with oxidizing agents produced heat-stable, high molecular weight aggregates of the permease on dodecyl sulfate gels, but no heat-stable dimer could be detected. The nonionic detergent Lubrol PX decreased the amount of dimer extractable at 30 °C with a concomitant increase in the monomeric form. These results suggest that the dimer depends predominantly on hydrophobic interactions for its stability and is not covalently cross-linked in that form by oxidizing agents.

The phosphotransferase sugar transport system (PTS) of *Escherichia coli* combines the transmembrane transport of its sugar substrates with an enzymatic reaction, the phosphorylation of these sugars using phosphoenolpyruvate (PEP). The sequence of PTS reactions and the contributing proteins have been well described [for reviews, see Postma & Lengeler (1985) and Saier (1985)]. The reactions are

$$\text{PEP} + \text{HPr} \overset{\text{enzyme I}}{\leftrightarrow} \text{HPr-P} + \text{pyruvate} \quad (1)$$

$$\text{HPr-P} + \text{sugar (out)} \xrightarrow[\text{(enzyme III)}]{\text{enzyme II}} \text{HPr} + \text{sugar-P (in)} \quad (2)$$

Enzyme I (EI) and HPr (a heat-stable protein) are soluble, generalized phosphocarrier proteins, while the enzymes II (EII's), which are integral membrane proteins, provide sugar specificity. Some sugars, such as glucose, also require the participation of an enzyme III in the terminal phosphotransfer reaction. EI, HPr, the enzymes III, and, most recently, the EII's specific for mannitol (EII^{Mtl}) and glucose (EII^{Glc}) have all been shown to be covalently phosphorylated during the phosphotransfer reactions shown above [reviewed in Postma & Lengeler (1985) and Saier (1985)]. Under the appropriate conditions, EII's alone can also carry out an exchange reaction called transphosphorylation (Saier, 1977):

$$[^{14}\text{C}]\text{sugar} + \text{sugar-P} \overset{\text{enzyme II}}{\leftrightarrow} [^{14}\text{C}]\text{sugar-P} + \text{sugar}$$

Of the PTS EII's, EII^{Mtl} (also called the mannitol permease) has been the most extensively studied to date. It has been purified to homogeneity (Jacobson et al., 1979), characterized (Jacobson et al., 1983b,c; Roossien et al., 1984; Roossien & Robillard, 1984a), and functionally reconstituted into proteoliposomes (Leonard & Saier, 1983). In addition, the gene coding for EII^{Mtl} (*mtl A*) has been cloned and sequenced (Lee & Saier, 1983), and models for its intramembrane structure have been proposed (Lee & Saier, 1983; Jacobson et al., 1983a). However, little is known as yet about the mechanism used by EII^{Mtl} to transport and phosphorylate its substrate, although a covalently phosphorylated form of the enzyme has been demonstrated (Roossien & Robillard, 1984b; Waygood et al., 1984).

Recently, Roossien and Robillard (1984b) have shown that the phosphorylated form of EII^{Mtl} is capable of forming a dimer in membrane vesicles. Dimers were identified in these studies by labeling of *E. coli* membranes with [^{32}P]PEP in the presence of EI and HPr and extraction of EII^{Mtl} from the membrane with sodium dodecyl sulfate at 30 °C. In this paper, we have used a minicell labeling technique (Lee et al., 1981) to monitor dimer formation by the total complement (unphosphorylated and phosphorylated) of EII^{Mtl} molecules. Factors that affect the amount of extractable dimer are identified and are correlated with EII^{Mtl} activities. Our results suggest that the monomer–dimer equilibrium could by physiologically relevant in controlling the activities of EII^{Mtl}.

Materials and Methods

Labeling of EII^{Mtl}. Minicells of *E. coli* strain MV1009 containing the plasmid pLC15-48, which encodes the mannitol operon, were isolated as previously described by Lee et al. (1981). EII^{Mtl} was selectively labeled by incubation of the minicells with [^{35}S]methionine as described by Lee et al. (1981) with the inclusion of 1 mM phenylmethanesulfonyl fluoride (PMSF) to inhibit proteolysis. The minicells were then washed 3 times (12000*g*, 10 min) with 30 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.0,

† This research was supported by USPHS Grant GM28226 from the National Institute of General Medical Sciences to G.R.J. and a Grant-in-Aid of Research from Sigma Xi Scientific Research Society to M.M.S.

* Author to whom correspondence should be addressed.

0006-2960/86/0425-4046$01.50/0

10 mM sodium ethylenediaminetetraacetate (NaEDTA), and 1 mM PMSF and frozen overnight at −70 °C. After being thawed, the minicells were lysed in a French pressure cell at 15000 psi in the same buffer with the addition of 1 mM dithiothreitol to produce predominantly everted membrane vesicles (M. Stephan and G. Jacobson, unpublished observations). After a low-speed centrifugation (3000*g*, 5 min) to remove unbroken minicells, the vesicles were collected and washed once by centrifugation at 100000*g* for 2 h at 4 °C. The vesicles were resuspended in 30 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol, and 10 mM NaEDTA (TDE buffer) plus 1 mM PMSF. Protein concentration was estimated according to the method of Lowry et al. (1951).

Electrophoresis and Autoradiography. Labeled EII^{Mtl} was extracted from minicell membrane vesicles in 62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 10% glycerol, and 0.003% bromphenol blue for 30 min at 30 °C. This buffer was routinely prepared at 3-fold the concentration and added to each sample at one-third the final volume. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed as described by Laemmli (1970), with a 1.5-mm, 9% resolving gel. The gel was soaked in 1 M sodium salicylate, pH 8.0, at room temperature for 15 min to enhance autoradiography and immediately dried (Chamberlain, 1979). Prior to enhancement, lanes containing molecular weight markers were cut off and stained separately with Coomassie brilliant blue. Autoradiography on Kodak XAR-5 X-ray film required 1–7-day exposures at −70 °C, depending upon the amount of sample applied. Labeled bands were quantitated by using a Zeineh soft laser scanning densitometer.

EII^{Mtl} Assays. Assays of mannitol 1-phosphate and PEP-dependent phosphorylation of D-mannitol catalyzed by EII^{Mtl} were performed as described previously (Jacobson et al., 1983a), except that [^{3}H]mannitol was used in experiments employing minicell membranes to reduce the background due to the [^{35}S]methionine label. A cytoplasmic fraction from *Salmonella typhimurium*, strain LJ144, containing elevated levels of EI and HPr, was also prepared as described previously (Begley et al., 1982). This fraction was used in some experiments to catalyze the phosphorylation of EII^{Mtl} prior to electrophoresis, as well as in assays of EII^{Mtl} activity and in measurements of EII^{Mtl}-P concentration. For use in electrophoresis and measurement of EII^{Mtl}-P levels, the EI–HPr preparation was first dialyzed overnight at 4 °C to remove endogenous PEP and other small molecules. EII^{Mtl} was purified in Lubrol PX as described previously (Jacobson et al., 1983b).

Effects of Substrates, Products, and Lubrol PX. Effects of substrates, products, and Lubrol PX on the amount of EII^{Mtl} dimer extracted were assessed by preincubating labeled minicell membranes (0.72 mg/mL) with these factors for 10 min at 30 °C prior to electrophoresis (see Table I for concentrations), except for those samples containing the EI–HPr preparation, which are described below. After preincubation, half the volume of 3-fold-concentrated extraction buffer was added to each sample, and all samples were extracted and electrophoresed as described above.

The samples containing EI and HPr were treated differently in order to remove the EI–HPr preparation before extraction. Labeled minicell membranes (20 μL, final protein concentration = 0.56 mg/mL) were preincubated with 5 μL of EI–HPr preparation, plus 5 μL of 70 mM PEP and 5 μL of H_2O. Samples (7 μL each) were removed from the mixture at 5, 10, 20, and 30 min after the addition of PEP and immediately diluted 50-fold with TDE buffer. As a control, another 4-μL sample of membranes was preincubated for 30 min at 30 °C with the same amount of EI–HPr but without PEP and also diluted 50-fold with TDE buffer. Samples were centrifuged at 12800*g* for 30 min and the membrane pellets resuspended in 7 μL of TDE buffer each. One sample, a duplicate of the 30-min time point containing EI, HPr, and PEP, was further incubated with 40 mM mannitol for 10 min at 30 °C. These samples were extracted with 3.5 μL of 3-fold extraction buffer and electrophoresed as described above.

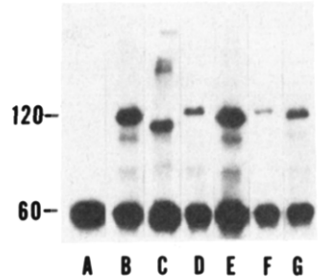

FIGURE 1: Effects of extraction temperature, Lubrol PX, and substrates on the amount of EII^{Mtl} dimer appearing on SDS–PAGE gels. [^{35}S]Methionine-labeled minicell membranes were preincubated, electrophoresed, and autoradiographed as described under Materials and Methods. Molecular weights were determined from protein standards run concurrently and are reported in kilodaltons. Lanes: (A) 100 °C; (B) 30 °C; (C–G) 30 °C; (C) 0.1% Lubrol PX; (D) EI, HPr, and 10 mM PEP; (E) EI and HPr; (F) EI, HPr, and 10 mM PEP followed by 40 mM mannitol; (G) 40 mM mannitol.

Measurement of EII^{Mtl}-P. The time course of EII^{Mtl}-P formation was measured directly under the same conditions as those used for incubation prior to extraction for electrophoresis. After resuspension, samples were incubated with 10 μM [^{3}H]mannitol (19.1 Ci/mmol) at 37 °C for 30 min. The product, [^{3}H]mannitol 1-phosphate, was then collected on Dowex 1-X2 columns (chloride form), washed with 25 column volumes of H_2O, eluted with 1 M LiCl, and counted in Triton X-100 containing scintillation fluid.

RESULTS

Subunit Interactions of EII^{Mtl}. Everted membrane vesicles isolated from labeled *E. coli* minicells showed a single major radioactive band on autoradiograms of SDS–PAGE gels when the proteins were extracted with SDS at the usual temperature of 100 °C. This band corresponded to the EII^{Mtl} monomer, M_r 60000 (Figure 1, lane A). However, mild SDS extraction conditions (30 °C for 30 min) led to the appearance of a second band, M_r 120000 (Figure 1, lane B). This band, presumably a dimer of EII^{Mtl}, could be partially dissociated by preincubating the membranes with the nonionic detergent Lubrol PX prior to extraction from the membrane with SDS (Figure 1, lane C). Conditions that lead to the phosphorylation of EII^{Mtl}, i.e., the presence of the other PTS enzymes, EI and HPr, and the phospho donor PEP, significantly decreased the amount of dimer extracted from the membrane (Figure 1, lane D). In these experiments, EI and HPr were removed by 50-fold dilution and centrifugation, so that this effect was probably not due to competition for SDS in the extraction buffer. Also, EI plus HPr alone showed little or no effect (Figure 1, lane E). The subsequent addition of mannitol, which results in turnover of phosphorylated EII^{Mtl} (Roossien & Robillard, 1984b), resulted in even less dimer in the extracted fraction (Figure 1, lane F). Mannitol alone also had a dissociatory effect on the dimer (Figure 1, lane G). These results are quantitated in Table I, which also shows that PEP alone, as well as the products of the phosphorylation/dephosphorylation reaction, pyruvate and mannitol 1-phosphate,

Table I: Quantitative Effects of Substrates, Products, and Lubrol PX on EII^{Mtl} Dimer[a]

condition	% dimer[b]
control	33 ± 1
0.1% Lubrol PX	20 ± 1
0.25% Lubrol PX	13 ± 1
EI + HPr[c]	33 ± 2
EI + HPr + 10 mM PEP[c]	15 ± 2
EI + HPr + 10 mM PEP, followed by 40 mM mannitol[c]	9 ± 4
40 mM mannitol	19 ± 1
10 mM PEP	31 ± 1
10 mM pyruvate	30 ± 1
10 mM mannitol 1-phosphate	32 ± 1

[a] Preincubations performed for 10 min at 30 °C (unless otherwise noted) as described under Materials and Methods. [b] Mean percent dimer relative to total monomer plus dimer quantitated by densitometry of two to six gels for each condition (±SD). [c] Preincubation for 30 min at 30 °C as described under Materials and Methods.

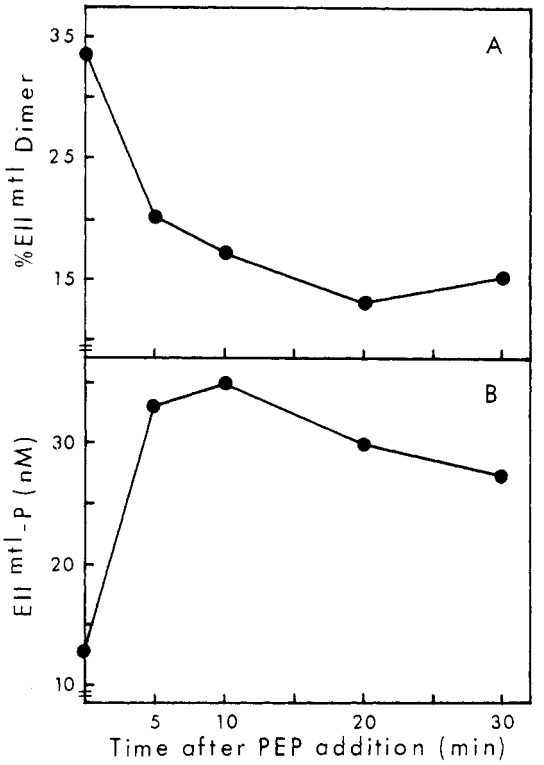

FIGURE 2: (A) Time course of EII^{Mtl} dimer dissociation in the presence of EI, HPr, and 10 mM PEP. [^{35}S]Methionine-labeled minicell membranes were preincubated with EI, HPr, and PEP and then extracted, electrophoresed, and autoradiographed as described under Materials and Methods. Bands were quantitated by densitometry, and percent dimer relative to total monomer + dimer was calculated. (B) Time course of EII^{Mtl}-P formation. Minicell membranes were preincubated under conditions identical with those used in (A), and the amount of EII^{Mtl}-P formed was measured as described under Materials and Methods. The 0-min time point represents the apparent concentration of EII^{Mtl}-P present in the absence of EI, HPr, and PEP (13 nM).

had little or no effect on the amount of dimer extracted.

The dissociatory effect of EI, HPr, and PEP together is shown as a function of time in Figure 2A. In order to show that this effect was accompanied by the phosphorylation of EII^{Mtl}, it was necessary to directly measure EII^{Mtl}-P concentration under these conditions. These results, shown in Figure 2B, demonstrate that phosphorylation of EII^{Mtl} occurred with a time course similar to that seen for the dissociation of EII^{Mtl} dimer. The highest concentration of EII^{Mtl}-P measured in this fashion was 35 nM (Figure 2B, 10-min time point), which compares with the approximate total concentration of EII^{Mtl} present in the minicell membranes, 43 nM, calculated from

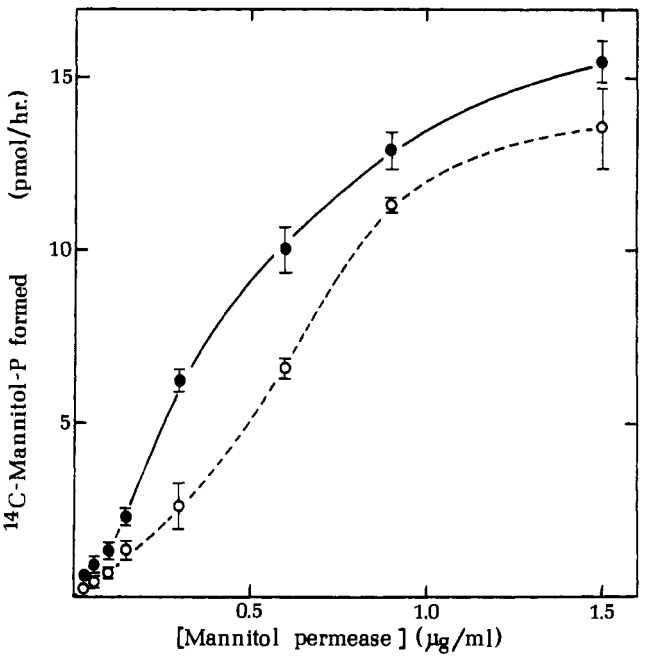

FIGURE 3: Activation of transphosphorylation by inorganic phosphate as a function of purified EII^{Mtl} concentration. Transphosphorylation was measured as referred to under Materials and Methods except that the buffer (pH 7.0) used was 0.1 M K-HEPES (○) or 0.1 M potassium phosphate (●). The data points shown represent the means and standard deviations of at least three independent determinations for each concentration of EII^{Mtl}. Each assay mixture contained Lubrol PX at a constant concentration of 0.25%.

the specific activity of EII^{Mtl} in this preparation (Jacobson et al., 1983b). These results also show that in the absence of EI, HPr, and PEP, >63% of the total EII^{Mtl} pool in minicell membranes was in the unphosphorylated state (cf. legend to Figure 2).

Effect of Inorganic Phosphate on Subunit Association. It has been shown that the transphosphorylation activity of purified EII^{Mtl} is stimulated at high enzyme concentrations (Roossien et al., 1984; Leonard & Saier, 1983). This activation was proposed by these workers to be due to enhanced subunit association of EII^{Mtl} monomers and taken as indirect evidence that this activity, at least, depends on the formation of an EII^{Mtl} dimer. Figure 3 (lower curve) shows the same type of sigmoidal dependence of EII^{Mtl} transphosphorylation activity on enzyme concentration reported by these groups. The buffer used in these experiments was 0.1 M potassium *N*-(2-hydroxyethyl)piperazine-*N'*-ethanesulfonate (K-HEPES), pH 7.0. However, when the same experiment was performed in 0.1 M potassium phosphate buffer at the same pH, the curve was less sigmoidal (Figure 3, upper curve). A comparison of the two curves in Figure 3 shows that the effect of phosphate was to activate EII^{Mtl} transphosphorylation activity about 2-fold at low enzyme concentrations, while little activation was apparent at higher enzyme concentrations. A simple explanation for this effect could be that inorganic phosphate activates transphosphorylation by promoting subunit association of EII^{Mtl}.

The potential importance of subunit interactions in the transphosphorylation activity of EII^{Mtl} was therefore more directly investigated by testing the effects of increasing phosphate concentration on both transphosphorylation activity and the amount of dimer extracted with minicell membrane vesicles. Figure 4 shows that, as inorganic phosphate was increased from 10 to 100 mM, transphosphorylation activity and the amount of dimer extracted increased in parallel, up

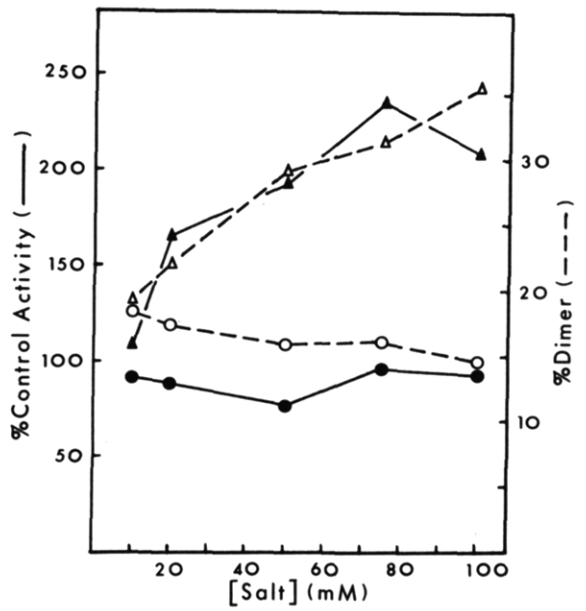

FIGURE 4: Effects of inorganic phosphate on EII^{Mtl} transphosphorylation activity (—) and the amount of EII^{Mtl} dimer appearing on autoradiograms of SDS–PAGE gels (– –). [^{35}S]Methionine-labeled minicell membranes (7 μL) were preincubated with sodium phosphate (△) or Na-HEPES (○) (pH 7.0) at the concentrations shown for 10 min at 30 °C, in the presence of 0.1% Lubrol PX (total volume = 10 μL, protein concentration = 0.60 mg/mL). A 3-μL aliquot was removed from each sample, extracted with 1.5 μL of 3-fold extraction buffer, electrophoresed, and quantitated as described under Materials and Methods. Each data point (– –) represents the mean percent dimer relative to monomer + dimer from three gels. The remainder of each sample was assayed for transphosphorylation activity as referred to under Materials and Methods, except that the buffer used was the same as the preincubation buffer for each sample. Each data point (—) represents the mean percent control (no salt) activity of three independent assays. (○, ●) Na-HEPES; (△, ▲) sodium phosphate.

to a maximum value of about 2-fold over the control values. This effect was not seen with the control salt, Na-HEPES. Lubrol PX (0.1%) was included in both the extraction and assay buffers in these experiments to allow for a more direct comparison with the results shown in Figure 3.

Effect of Oxidizing Agents. Other investigators have suggested that EII^{Mtl} monomers might associate by the oxidation of sulfhydryl groups to form an intermolecular disulfide bond (Roossien & Robillard, 1984a). If such a covalent bond were involved in EII^{Mtl} dimerization, then under oxidizing conditions it might be possible to demonstrate the formation of a dimer that is stable to SDS at 100 °C. However, SDS extraction of membranes before or after treatment with several concentrations of either of the oxidizing reagents $K_3[Fe(CN)_6]$ or $CuSO_4$ did not result in the formation of a heat-(100 °C) stable dimer (Table II). Although dimer was detectable at 30 °C at the lowest concentration of $CuSO_4$ and at all concentrations of ferricyanide, dimer was absent in the same samples extracted at 100 °C with a concomitant increase in monomer compared to extraction at 30 °C (Table II). At a given oxidant concentration, however, the amount of aggregate was virtually the same at 30 and 100 °C. These results indicate that most of the aggregate appearing at 30 °C was covalently cross-linked, while none of the dimers extracted at this temperature were linked by disulfide bonds. Finally, gradually increasing the ferricyanide concentration followed by extraction at 30 °C showed that, although the proportion of dimer to monomer did increase at lower concentrations, this effect appeared to be due to an increase in aggregate at the expense of the monomer, while the total amount of dimer remained unchanged at about 33%. At the higher concentrations, a loss of dimer to aggregate also occurred (Figure 5).

Table II: Effects of Oxidizing Agents on EII^{Mtl} Subunit Interactions[a]

condition	extraction temp (°C)	% aggregate[b]	% dimer[b]	% monomer[b]
control	30	9	27	64
	100	2	0	98
$CuSO_4$				
10 mM	30	29	15	56
	100	28	0	72
25 mM	30	100	0	0
	100	100	0	0
100 mM	30	100	0	0
	100	100	0	0
$K_3[Fe(CN)_6]$				
10 mM	30	12	20	68
	100	16	0	84
50 mM	30	33	18	49
	100	28	0	72
100 mM	30	47	10	44
	100	43	0	57

[a] Minicell membranes (8 μL) containing ^{35}S-labeled EII^{Mtl} were preincubated under the conditions shown (final volume 10 μL) for 5 min at 30 °C. Final protein concentration was 0.68 mg/mL. Each sample was then divided in half and 3 μL of 3-fold-concentrated extraction buffer added to each half. One of each pair was extracted at 30 °C for 30 min and the other at 100 °C for 5 min. Samples were electrophoresed, and the bands were quantitated as described under Materials and Methods. [b] Percent relative to total aggregate + dimer + monomer quantitated by densitometry.

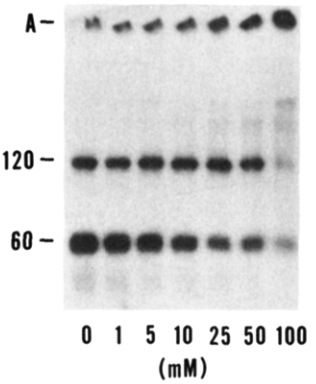

FIGURE 5: Effect of increasing concentrations of $K_3[Fe(CN)_6]$ on the amounts of EII^{Mtl} aggregate, dimer, and monomer extracted at 30 °C. [^{35}S]Methionine-labeled minicell membranes (3 μL) were preincubated with the concentrations of $K_3[Fe(CN)_6]$ shown for 5 min at 30 °C (final volume = 4 μL, protein concentration = 0.38 mg/mL). The samples were extracted at 30 °C with 2 μL of 3-fold extraction buffer, electrophoresed, and autoradiographed as described under Materials and Methods. Molecular weights were determined from protein standards run concurrently and are reported in kilodaltons. (A = high molecular weight aggregate).

Discussion

A dimeric form of EII^{Mtl} was first demonstrated by Roossien and Robillard (1984b), who found that this form of the enzyme was detectable when *E. coli* membranes were extracted with SDS under mild conditions. These investigators used [^{32}P]PEP to covalently label the protein for visualization on autoradiograms, thus allowing only the phosphorylated form of EII^{Mtl} to be observed. With the minicell system, however, we were able to examine the entire EII^{Mtl} pool, including the unphosphorylated form, since the protein was labeled during its synthesis with [^{35}S]methionine.

Our results show that approximately 30% of the total amount of EII^{Mtl} can be extracted as a dimer from minicell

membranes under conditions similar to those used by Roossien and Robillard (1984b). Conditions that lead to the phosphorylation of EII^{Mtl} resulted in less dimer extracted from the membranes (Figures 1 and 2, Table I). This dissociatory effect took place in a time-dependent manner (Figure 2A), and phosphorylation of the enzyme followed a similar time course (Figure 2B). Interestingly, mannitol alone decreased the amount of extractable dimer whether the enzyme was phosphorylated or not (Figure 1 and Table I). These results, taken together, suggest that conditions that favor the PEP-dependent phosphorylation of mannitol by EII^{Mtl} (i.e., phosphorylation and the presence of the sugar substrate) destabilize the dimeric form of the enzyme, at least after extraction with detergent. Therefore, it is possible that either the monomer or a less stable conformation of the dimer (compared to unphosphorylated EII^{Mtl}) participates at least transiently in the PEP-dependent phosphorylation of mannitol. Alternatively, phosphorylation and/or turnover of EII^{Mtl} could only affect the stability of the dimer in SDS, and not its stability in the membrane before extraction. Techniques for investigating the monomer–dimer equilibrium directly in the membrane will be necessary to decide between these alternatives.

On the basis of their experiments, Roossien and Robillard (1984b) suggested that the dimeric form of EII^{Mtl} may be essential for its activity. Although this appears to be true for transphosphorylation (see below), our results leave open the possibility that the PEP-dependent reaction could be catalyzed by a monomer. Although more ^{32}P label appeared in the dimer than in the monomer in the experiments of Roossien and Robillard (1984b), their results are not necessarily inconsistent with ours. For example, if the phospho group on the extracted form of the monomer were more labile to hydrolysis than the same group(s) on the dimer, and if the unphosphorylated form of the monomer so produced reassociates slowly, if at all, in the presence of SDS, then both sets of results could be explained. Further experiments will be required, however, to clarify this apparent discrepancy.

In contrast to the results described above for the PEP-dependent reaction, our experiments suggest that a dimeric form of EII^{Mtl} is necessary, or is at least more active than the monomer, in catalyzing the mannitol–mannitol 1-phosphate transphosphorylation reaction. Previous investigators (Leonard & Saier, 1983; Roossien et al., 1984) had shown that the transphosphorylation activity of purified EII^{Mtl} rises nonlinearly with increasing concentrations of the enzyme. This result suggested that subunit interactions, which would be favored at higher enzyme concentrations, could be important for this activity. We have shown here that inorganic phosphate stimulates this activity of the purified enzyme only at low enzyme concentrations, suggesting that its effect is to promote the necessary subunit interactions (Figure 3). This conclusion is supported by the experiments shown in Figure 4. When compared with a control buffer, Na-HEPES, increasing concentrations of inorganic phosphate activated transphosphorylation and increased the amount of dimer in parallel.

The EII^{Mtl} dimer was partially dissociated by the nonionic detergent Lubrol PX (Figure 1, Table I). This result suggests that primarily hydrophobic interactions are important in the subunit interaction as proposed by Roossien and Robillard (1984b). The EII^{Mtl} dimer extracted at 30 °C does not seem to involve an intermolecular disulfide bond, however, as has been suggested for the purified enzyme on the basis of stoichiometric measurements with irreversible sulfhydryl reagents (Roossien & Robillard, 1984a). Such a covalently linked dimer should not be dissociated by SDS at 100 °C. Extraction of minicell membranes in the presence of oxidizing agents such as $K_3[Fe(CN)_6]$ [which has been shown to oxidize and inactivate EII^{Mtl} (Roossien & Robillard, 1984a; Grenier et al., 1985)] and $CuSO_4$ failed to result in a heat-(100 °C) stable dimer, although dimer was detected at the lower concentrations of these compounds when membranes were extracted at 30 °C (Table II). Rather, the effect of increasing concentrations of oxidizing agents was to increase the amount of high molecular weight aggregate at the expense of EII^{Mtl} monomer, without the intermediate formation of a heat-stable dimer (Figure 5 and Table II). Therefore, if a covalent dimer is formed under oxidizing conditions, it must be converted to the aggregated form too rapidly to be observed by this technique. It should also be pointed out that the aggregated form of EII^{Mtl} could also contain other unlabeled proteins present in the preparation that are cross-linked to EII^{Mtl} during oxidation.

The experiments presented in this paper show that the substrate mannitol, PEP-dependent phosphorylation, inorganic phosphate, and oxidizing agents all influence subunit interactions of EII^{Mtl}. It should be pointed out that these interactions were assessed in SDS extracts of *E. coli* membranes. Therefore, it is possible that within the membrane EII^{Mtl} could exist entirely as a dimer under all conditions and the effects we observed could simply reflect the stability of this dimer to SDS extraction. Alternatively, it is equally possible that a monomer–dimer equilibrium does exist within the membrane and that these factors directly influence this equilibrium. Further work will be necessary to decide between these possibilities. In either case, however, our results show that subunit interactions are likely to be important in controlling the catalytic activities of EII^{Mtl}.

Added in Proof

Recently, Roossien et al. (1986) have obtained evidence for a dimer of purified EII^{Mtl} using bifunctional sulfhydryl reagents. Interestingly, EII^{Mtl}-P was poorly cross-linked by these compounds compared to the unphosphorylated form. This indicates either that the vicinal dithiols that are cross-linked are less reactive in EII^{Mtl}-P or that phosphorylation of EII^{Mtl} promotes dissociation as proposed in this paper.

Acknowledgments

We thank Gail Begley for performing the experiments reported in Figure 3 of this paper.

Registry No. PEP, 138-08-9; PO_4^{3-}, 14265-44-2; pyruvate, 127-17-3; D-mannitol 1-phosphate, 15806-48-1; D-mannitol, 69-65-8; Lubrol PX, 9002-92-0; mannitol permease, 91386-44-6; phosphohistidinoprotein–hexose phosphotransferase, 37278-09-4.

References

Begley, G. S., Hansen, D. E., Jacobson, G. R., & Knowles, J. R. (1982) *Biochemistry 21*, 5552–5556.

Chamberlain, J. P. (1979) *Anal. Biochem. 98*, 132–135.

Grenier, F. C., Waygood, E. B., & Saier, M. H., Jr. (1985) *Biochemistry 24*, 47–51.

Jacobson, G. R., Lee, C. A., & Saier, M. H., Jr. (1979) *J. Biol. Chem. 254*, 249–252.

Jacobson, G. R., Kelly, D. M., & Finlay, D. R. (1983a) *J. Biol. Chem. 258*, 2955–2959.

Jacobson, G. R., Lee, C. A., Leonard, J. E., & Saier, M. H., Jr. (1983b) *J. Biol. Chem. 258*, 10748–10756.

Jacobson, G. R., Tanney, L. E., Kelly, D. M., Palman, K. B., & Corn, S. B. (1983c) *J. Cell. Biochem. 23*, 231–240.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Lee, C. A., & Saier, M. H., Jr. (1983) *J. Biol. Chem. 258*, 10761–10767.
Lee, C. A., Jacobson, G. R., & Saier, M. H., Jr. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 7336–7340.
Leonard, J. E., & Saier, M. H., Jr. (1983) *J. Biol. Chem. 258*, 10757–10760.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Postma, P. W., & Lengeler, J. W. (1985) *Microbiol. Rev. 49*, 232–269.
Roossien, F. F., & Robillard, G. T. (1984a) *Biochemistry 23*, 211–215.
Roossien, F. F., & Robillard, G. T. (1984b) *Biochemistry 23*, 5682–5685.
Roossien, F. F., Blaauw, M., & Robillard, G. T. (1984) *Biochemistry 23*, 4934–4939.
Roossien, F. F., van Es-Spiekman, W., & Robillard, G. T. (1986) *FEBS Lett. 196*, 284–290.
Saier, M. H., Jr. (1977) *Bacteriol. Rev. 41*, 856–871.
Saier, M. H., Jr. (1985) *Mechanisms and Regulation of Carbohydrate Transport in Bacteria*, Academic, New York.
Waygood, E. B., Mattoo, R. L., & Peri, K. G. (1984) *J. Cell. Biochem. 25*, 139–159.

Molecular Structure of Rat Brain Apamin Receptor: Differential Photoaffinity Labeling of Putative K^+ Channel Subunits and Target Size Analysis†

Michael J. Seagar,*,‡ Catherine Labbé-Jullié,‡ Claude Granier,‡ Alexandra Goll,§ Hartmut Glossmann,§ Jurphaas Van Rietschoten,‡ and François Couraud‡

Laboratoire de Biochimie, Faculté de Médecine, Secteur Nord, Unité 172, Institut National de la Santé et de la Recherche Médicale, 13326 Marseille Cedex 15, France, and Institut fur Biochemische Pharmakologie, 6020 Innsbruck, Austria

Received November 15, 1985; Revised Manuscript Received February 10, 1986

ABSTRACT: Two photoreactive apamin derivatives were prepared with an aryl azide [[(azidonitrophenyl)amino]acetate (ANPAA)] group coupled at different positions on the neurotoxin molecule. These ligands were used to identify membrane components in the environment of the neuronal binding site that is associated with a Ca^{2+}-activated K^+ channel. ^{125}I-[α-ANPAA-Cys_1]apamin labeled a single M_r 86 000 chain in cultured neurons whereas two bands corresponding to M_r 86 000 and 59 000 were detected in synaptic membrane preparations, suggesting that the M_r 59 000 polypeptide may be a degradation product. ^{125}I-[ϵ-ANPAA-Lys_4]apamin however incorporated uniquely into two smaller components with M_r 33 000 and 22 000 in both cultured neurons and synaptic membranes. Randomly modified ^{125}I-ANPAA-apamin gave a cross-linking profile equivalent to the sum of those obtained with the two defined derivatives. The apamin binding site seems to be located at the frontier between three or more putative K^+ channel subunits which are only accessible from limited regions of the receptor-associated photoprobe. Irradiation of frozen rat brain membranes with high-energy electrons led to a reduction in ^{125}I-apamin receptor capacity, yielding a target size for the functional binding unit of M_r 84 000–115 000, which could be constituted by the M_r 86 000 subunit alone or by the M_r 86 000 subunit in conjunction with one of the two smaller subunits.

Considerable advances in the molecular characterization of ion channel proteins, responsible for action potential generation in excitable cells, have been achieved in recent years. The purification and functional reconstitution of the voltage-sensitive Na^+ channel have been accomplished with radiolabeled neurotoxins as biochemical probes (Catterall, 1984). Similarly, components of the voltage-dependent Ca^{2+} channel have been isolated (Curtis & Catterall, 1984), in association with receptors for labeled Ca^{2+} antagonists (Glossmann & Ferry, 1985). This type of approach has not been widely applicable to K^+ channels due to an almost total lack of specific toxins or drugs that can be used in ligand binding assays.

Apamin, a 2000-dalton peptide purified from bee venom, seems at the present time to be an exception. Nanomolar concentrations of apamin specifically block a K^+ permeability, present in a variety of cell types, that is activated by an increase in the intracellular free Ca^{2+} concentration. In neuroblastoma and skeletal muscle cells it inhibits a macroscopic slow K^+ current which underlies the long-lasting after hyperpolarization (Hugues et al., 1982b; Romey & Lazdunski, 1984; Cognard et al., 1984). However, the tetraethylammonium-sensitive Ca^{2+}-activated K^+ conductance, which has been extensively studied by single-channel recording techniques, is not blocked by apamin (Romey & Lazdunski, 1984). This apparent contradiction has been clarified by recent studies in sympathetic ganglia that indicate that two types of Ca^{2+}-activated K^+ current coexist in the same neuron, each having a distinct physiological role. The fast current which is blocked by tetraethylammonium contributes to spike repolarization whereas the apamin-sensitive slow current produces the prolonged hyperpolarization which may modulate repetitive firing characteristics (Pennefather et al., 1985). Recent work has involved the use of apamin and its derivatives in an attempt to gain an insight into the structure of this neuronal K^+ channel.

We have detected high-affinity binding sites for mono-[^{125}I]iodoapamin on primary cultured neurons and have correlated receptor occupancy to an inhibition of ion efflux

† This research was supported by grants from the Centre National de la Recherche Scientifique (U.A. 553) and the Institut National de la Santé et de la Recherche Médicale (U. 172).
* Address correspondence to this author.
‡ Institut National de la Santé et de la Recherche Médicale.
§ Institut fur Biochemische Pharmakologie.

0006-2960/86/0425-4051$01.50/0