Relation between the Oligomerization State and the Transport and Phosphorylation Function of the Escherichia coli Mannitol Transport Protein: Interaction between Mannitol-Specific Enzyme II Monomers Studied by Complementation of Inactive Site-Directed Mutants†

Harry Boer, Ria H. ten Hoeve-Duurkens, and G. T. Robillard*

The Groningen Biomolecular Science and Biotechnology Institute (GBB) and the Department of Biochemistry, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

Received May 8, 1996; Revised Manuscript Received July 25, 1996

ABSTRACT: Previous experiments with the mannitol-specific enzyme II of Escherichia coli, EII\textsuperscript{mtl}, have demonstrated that (1) the enzyme is a dimer, (2) the dimer is necessary for maximum activity, and (3) phosphoryl groups could be transferred between EII\textsuperscript{mtl} subunits [van Weeghel et al. (1991) Biochemistry 30, 1768–1773; Weng et al. (1992) J. Biol. Chem. 267, 19529–19535; Weng & Jacobson (1993) Biochemistry 32, 11211–11216; Stolz et al. (1993) J. Biol. Chem. 268, 27094–27099]. The experiments in this article address the mechanistic role of the dimer. They indicate that the A, B, and C domains of EII\textsuperscript{mtl} preferentially interact within the same subunit. Site-directed mutants in each of the three domains of EII\textsuperscript{mtl} were used to study phosphoryl group transfer by the EII\textsuperscript{mtl} dimer in vitro and mannitol transport in vivo. The C domain mutant, EII\textsuperscript{mtl}(G196D), which was unable to bind mannitol, and the separated C domain, IIC\textsuperscript{mtl}, which was unable to phosphorylate mannitol, formed a heterodimer which was capable of mannitol phosphorylation in vitro and mannitol transport in vivo. The rates of phosphorylation were approximately 10-fold lower in heterodimers containing two inactive subunits relative to the rates in heterodimers containing one inactive and one wild type subunit; phosphoryl group transfer through one subunit is kinetically preferred to intersubunit transfer. Heterodimers formed in vivo between one wild type EII\textsuperscript{mtl} subunit and the CB domain double mutant, EII\textsuperscript{mtl}(G196D/C384S), transported mannitol as rapidly as wild type EII\textsuperscript{mtl} alone; the presence of the inactive double mutant subunit did not reduce the transport rate. Thus, only one active A, B, and C domain in the dimer is sufficient for transport and phosphorylation activity, and if all three domains are situated on the same subunit, maximum rates are achieved.

Uptake and phosphorylation of the carbohydrate, mannitol, in Escherichia coli is catalyzed by the mannitol transport protein, enzyme II\textsuperscript{mtl}, which is located in the cytoplasmic membrane. EII\textsuperscript{mtl} is a member of the phosphoenolpyruvate-dependent phosphotransferase system (PTS) of E. coli (Lolkema & Robillard, 1992; Postma et al., 1993). Transport proteins belonging to this system are group translocation enzymes are general PTS proteins; all sugar-specific EII's are phosphorylated by P-HPr.

The mannitol transport protein consists of three domains, a membrane-bound C domain and two cytoplasmic domains, B and A (van Weeghel et al., 1991; Robillard et al., 1993). The A domain is phosphorylated at His554 by P-HPr. The phosphoryl group is transferred from His554 to Cys384 in the B domain, the second phosphorylation site on EII\textsuperscript{mtl}. The phosphorylation of the mannitol transport protein is catalyzed by the mannitol transport protein, enzyme II\textsuperscript{mtl}, which is located in the cytoplasmic membrane. EII\textsuperscript{mtl} is a member of the phosphoenolpyruvate-dependent phosphotransferase system (PTS) of E. coli (Lolkema & Robillard, 1992; Postma et al., 1993). Transport proteins belonging to this system are group translocation enzymes are general PTS proteins; all sugar-specific EII's are phosphorylated by P-HPr. The mannitol transport protein consists of three domains, a membrane-bound C domain and two cytoplasmic domains, B and A (van Weeghel et al., 1991; Robillard et al., 1993). The A domain is phosphorylated at His554 by P-HPr. The phosphoryl group is transferred from His554 to Cys384 in the B domain, the second phosphorylation site on EII\textsuperscript{mtl} (Scheme 1) (Pas et al., 1988). The phosphoryl group is then transferred to mannitol which is translocated by the membrane-bound C domain (Grisafi et al., 1989; Lolkema et al., 1990).

Scheme 1

\[
P-HPr + EII\textsuperscript{mtl} \rightarrow HPr + EII\textsuperscript{mtl}(H554)-P
\]

\[
EII\textsuperscript{mtl}(H554)-P \rightarrow EII\textsuperscript{mtl}(C384)-P
\]

\[
EII\textsuperscript{mtl}(C384)-P + mtl (out) \rightarrow EII\textsuperscript{mtl} + mtl-P (in)
\]

The association state of EII\textsuperscript{mtl} has been the focus of attention with different techniques for many years. (i) Physical size measurements indicate that EII\textsuperscript{mtl} is an oligomeric protein with a mass that is most likely to be a dimer (Pas et al., 1987; Kandekhar et al., 1989; Lolkema et al., 1993; Boer et al., 1994). (ii) Dimeric forms of EII\textsuperscript{mtl} and
the membrane-bound C domain were observed by gel electrophoresis after extraction of the protein out of the membrane (Rooszien & Robillard, 1984a; Stephan & Jacobson, 1986). (iii) EII\textsuperscript{mtl} monomers could also be specifically cross-linked via sulfhydryl groups using either bifunctional sulfhydryl reagents or oxidants (Rooszien & Robillard, 1984b; Rooszien et al., 1986). (iv) Kinetic studies on the wild type enzyme indicated the existence of oligomeric forms of EII\textsuperscript{mtl} (Saier et al., 1980; Leonard & Saier, 1983; Rooszien et al., 1984; Lolkema & Robillard, 1990), as did kinetics on inactive mutants of the first phosphorylation site, H554A, and the second phosphorylation site, C384S. A heterodimer consisting of these two mutants was capable of catalyzing both PEP-dependent phosphorylation and mtl/mtl-P exchange (van Weeghel et al., 1991; Weng et al., 1992). In vivo studies of Weng et al. (1992) have shown that, when these two phosphorylation site mutants are coexpressed in *E. coli*, growth on mannitol is possible. These complementation studies indicate that phosphoryl group transfer between EII\textsuperscript{mtl} subunits is possible. Phosphoryl group transfer between subunits was also demonstrated for the mannose transporter (Stolz et al., 1993).

The existence of EII\textsuperscript{mtl} dimers and the possibility of phosphoryl group transfer between subunits lead to the question of the role of the individual subunits and the dimer in mannitol phosphorylation and uptake. More specifically, which domains of EII\textsuperscript{mtl} interact and what are the resulting inter- and intramolecular phosphoryl group transfer routes? What is the importance of these subunit interactions for the phosphorylation and transport of mannitol? These questions were studied by in vivo and in vitro complementation of single and double mutants of EII\textsuperscript{mtl}. EII\textsuperscript{mtl} was mutated at the two phosphorylation sites on the cytoplasmic A and B domains and in the mannitol-binding, membrane-bound C domain, resulting in inactive mutants that show no mannitol binding and/or phosphorylation activity. PEP-dependent mannitol phosphorylation activity, mtl/mtl-P exchange activity, mannitol binding, and mannitol uptake activities were measured for the different mutant enzymes and combinations of enzymes.

**MATERIALS AND METHODS**

Materials. The oligonucleotides were synthesized on an Applied Biosystems Model 380B DNA synthesizer by Eurosequence bv. Groningen. M13K07 helper phage and the DNA-sequencing kit were obtained from Pharmacia. Klenow enzyme, restriction endonucleases, T4 DNA ligase, and T4 polynucleotide kinase were from Boehringer Mannheim. DecylPEG was synthesized by B. Kwant at the Department of Chemistry, University of Groningen. d-[1-\textsuperscript{14}C]Mannitol (2.04 GBq/mmol) was obtained from Amer sham. d-[1,3-H(N)]Mannitol (976.8 GBq/mmol) was from NEN Research Products, and mannitol 1-phosphate was purchased from Sigma. The GF/F glass microfiber filters were from Whatman. MacConkey agar was obtained from Difco. Enzyme I, enzyme II\textsuperscript{mtl}, HPr, and IIC\textsuperscript{mtl} were purified as described previously (Robillard et al., 1979, 1993; van Dijk et al., 1990; Boer et al., 1994).

Bacterial Strains, Plasmids, and Growth Conditions. The *E. coli* strain CJ236 dut1, ung1, thi-1, relA1/pCI105 (cam’ F’) was used to prepare single-stranded template DNA that contains uracil for site-directed mutagenesis (Kunkel et al., 1987). *E. coli* strain JM101 Δ(lac-proAB), supE, thi, [F’, traD36, proA+ B+, lacPZΔM15] was used for various DNA techniques (Yanish-Perron et al., 1985). The *E. coli* bacterial strain which contains a chromosomal deletion in the wild type mtlA gene, LGS322 F thi-1, hisG1, argG6, metB1, tonA2, supE44, rpsL104, lacY1, galT6, gatR49, gatA50, Δ(mtI’p), mtlD’, Δ(gutRMDBA-reca), was used for selection and expression of the mutants of EII\textsuperscript{mtl} (Grisafi et al., 1989).

Plasmid pMamtlA is the expression vector used to produce wild type EII\textsuperscript{mtl} and was also used as the starting vector in the site-directed mutagenesis procedure (van Weeghel et al., 1990). The H554A and C384S mutants of EII\textsuperscript{mtl} were described previously (van Weeghel et al., 1991). Plasmid pMaICP, used for the expression of IIC\textsuperscript{mtl} was described previously (Boer et al., 1994). Plasmid pJRDI187 was described previously (Davidson et al., 1987). Depending on the experiment, strains were grown either on minimal medium plus glucose or on LB medium (10 g of bacto-tryptone, 5 g of yeast extract, and 10 g of NaCl per liter). Antibiotics were included at concentrations of 25 µg/mL chloramphenicol or 100 µg/mL ampicillin or both, as needed.

**Construction of the EII\textsuperscript{mtl} Mutants.** The G196D mutation was made following the Kunkel mutagenesis procedure using primer 5′-GAGAAGATATCGTGGTT-3′ and plasmid pMamt-1A containing the mtlA gene behind the P\textsuperscript{mtl} promoter (Kunkel, 1985). The mutation in plasmid pMaG196D could be detected because a unique EcoRV restriction site was created in the mtlA gene. The mutated mtlA gene was sequenced by the method of Sanger et al. (1977) and was identical to the previously published sequence (Lee & Saier, 1983), except for the mutation introduced. Plasmid pMcG196D, which contains a chloramphenicol resistance gene, was made by replacing the pMa-5-8 vector part of plasmid pMaG196D with vector pMc5-8. Plating *E. coli* strain LGS322 containing this mutated plasmid on MacConkey agar plates containing 1% d-mannitol gave colonies that had a white, mannitol fermentation-negative, phenotype indicative of a defect in mannitol transport.

Plasmid pMaG196D/C384S was created by replacing the SnuBl-XbaI fragment of plasmid pMaG196D with the same fragment from plasmid pMaC384S that contains the C384S mutation. In the new plasmid, amino acids 196 and 384 are mutated; this could be checked by cutting the plasmid with EcoRV and NheI, because both mutations created a unique restriction site.

Overexpression of the mutants was achieved by insertion of the λ-P, promoter with the cI857 λ-repressor gene into the mutant plasmids. For this purpose, an EcoRI-Sall fragment containing the P, promoter and repressor gene was excised from pJRDI187 and ligated into the corresponding restriction sites of the plasmid. This strategy for obtaining overexpression was identical to that published previously for the wild type enzyme and IIC\textsuperscript{mtl} (van Weeghel et al., 1990; Boer et al., 1994). The resulting overexpression vector contained the mutant enzyme behind a tandem P\textsubscript{P\textsuperscript{mtl}} promoter.

Plasmid pJRDIIC was constructed for the in vivo complementation study. This plasmid contains the same tandem P\textsubscript{P\textsuperscript{mtl}} promoter and IIC\textsuperscript{mtl}-encoding part as the IIC\textsuperscript{mtl} overexpression plasmid pMaICP, (Boer et al., 1994), but it has a different origin of replication than the pMa/c5-8 derivatives and can be used in combination with pMcG196D for the
coexpression of II\textsuperscript{mut} and EI\textsuperscript{mut}-G196D in \textit{E. coli}. A BamHI fragment that encodes for II\textsuperscript{mut} from the overexpression plasmid pMaICP, was ligated into plasmid pJRD187, resulting in plasmid pJRD1IC.

\textbf{Expression, Preparation of Membrane Vesicles, and Purification of the Mutants.} \textit{E. coli} strain LGS322 with a mtl\textsuperscript{A} gene deletion was used for expression of the mutants of EI\textsuperscript{mut}. Membrane vesicles were prepared as described previously (Lolkema et al., 1990). Wild type and mutant EI\textsuperscript{mut} were purified as described (Robillard et al., 1993).

\textbf{PEP-Dependent Mannitol Phosphorylation and Mannitol/Mannitol 1-phosphate Exchange Assays.} The PEP-dependent phosphorylation kinetics of EI\textsuperscript{mut} and the mutant proteins were measured in 25 mM Tris-HCl (pH 7.6), 5 mM MgCl\textsubscript{2}, 5 mM DTT, 5 mM PEP, and 0.25\% decylPEG at 30 °C. The concentration of enzyme I, HPr, and labeled mannitol depended on the experiment. The samples were incubated for 10 min at 30 °C before the reaction was started with labeled mannitol. Details are given in the figure legends and the text. The volume of the assay mixture was 100 \(\mu\text{L}\). Four 20 \(\mu\text{L}\) samples were taken at various times and loaded onto Dowex AG1-X2 columns. A 10 \(\mu\text{L}\) sample was used to measure the total amount of radioactivity in the assay. The assay procedure has been described in detail by Robillard and Blaauw (1987).

Mannitol/mannitol 1-phosphate exchange assays were done at 30 °C in 25 mM Tris-HCl (pH 7.6), 5 mM MgCl\textsubscript{2}, 5 mM DTT, 0.25\% decylPEG, and a given concentration mannitol 1-phosphate. The exchange reaction was started with \(^{3}\text{H}\)-mannitol after incubation for 10 min at 30 °C. The assay volume and the sample size were the same as for PEP-dependent phosphorylation assays. The assay procedure has been described by Lolkema et al. (1990).

\textbf{Mannitol Uptake Measurements.} For mannitol uptake measurements, cells expressing the EI\textsuperscript{mut} derivative of interest were grown in the media specified in the text or figure legend until OD\textsubscript{600} = 1, harvested by centrifugation (10 min, 6000g), washed with 50 mM KP\textsubscript{i} buffer (pH 7.5), and resuspended to an OD\textsubscript{600} of 10 in the same buffer. For measuring mannitol uptake, 90 \(\mu\text{L}\) aliquots of cells with an OD\textsubscript{600} of 1 were prepared by diluting the cells with 50 mM KP\textsubscript{i} buffer (pH 7.5). The uptake measurement was started by adding 10 \(\mu\text{L}\) of 35 \(\mu\text{M}\) \(^{14}\text{C}\)-mannitol to these cells. After the appropriate time, the uptake was quenched by the rapid addition of 2 \(\text{mL}\) of ice-cold 50 mM KP\textsubscript{i} buffer (pH 7.5) containing 1 mM HgCl\textsubscript{2}. The cell suspension was then rapidly filtered through Whatman GF/F filters, and the vessel and the filter were washed twice with 2 \(\text{mL}\) of the quenching buffer. The cells on the filters were solubilized with scintillation liquid, and the amount of radioactivity in the cells was determined.

\textbf{Flow Dialysis.} The flow dialysis procedure of Lolkema et al. (1990) was used for measuring the \(K_{d}\) of the enzyme for mannitol. The buffer conditions were 25 mM Tris-HCl (pH 7.5), 5 mM MgCl\textsubscript{2}, 5 mM DTT, and 0.5\% decylPEG. The measurements were done at 25 °C, varying the mannitol concentration from 50 to 600 nM for the wild type enzyme.

\textbf{Protein Determinations.} Protein concentrations in the preparations were determined by the method of Lowry (1951) with BSA as the standard. The wild type and mutant EI\textsuperscript{mut} concentrations were quantitated by flow dialysis as the number of mannitol binding sites extrapolated from a Scatchard plot of mannitol binding or with the pyruvate-

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{SDS-PAGE gel showing the expression of mutant EI\textsuperscript{mut}-G196D/C384S in \textit{E. coli} strain JM101: lane 1, marker proteins of 94, 67, 43, 30, 20.1, and 14.4 kDa; lane 2, membranes of \textit{E. coli} strain JM101 cells that express chromosomally encoded wild type EI\textsuperscript{mut}; lane 3, membranes derived from \textit{E. coli} strain JM101 cells that overexpress mutant EI\textsuperscript{mut}-G196D; and lane 4, membranes derived from \textit{E. coli} strain JM101 cells that overexpress double mutant EI\textsuperscript{mut}-G196D/C384S. The position of EI\textsuperscript{mut} is indicated with an arrow. All the \textit{E. coli} JM101 cells used for the preparation of these membranes were grown on minimal medium containing glucose to achieve a low expression level of wild type EI\textsuperscript{mut}.}
\end{figure}

\section{RESULTS}

\textbf{Characterization of the EI\textsuperscript{mut}-G196D Mutant.} Mutant EI\textsuperscript{mut}-G196D had a white, mannitol fermentation-negative, phenotype on MacConkey plates with 1\% mannitol when expressed in the EI\textsuperscript{mut} deletion strain LGS322. Membrane vesicles of this strain applied on a SDS–polyacrylamide gel showed a clear band at the EI\textsuperscript{mut} position, indicating that the mutation had no large effect on the expression of the mutant (Figure 1, lane 3). Apparently, mutation G196D in the C domain results in an enzyme with impaired mannitol binding and/or transport activity. The mutant protein was purified using the same method employed for the wild type EI\textsuperscript{mut}, and the PEP-dependent mannitol phosphorylation and the mtl/mtl-P exchange kinetics of the purified EI\textsuperscript{mut}-G196D were examined. PEP-dependent phosphorylation was measured under saturating HPr (17.6 \(\mu\text{M}\)) and EI (165 nM) conditions with a mannitol concentration ranging from 1–16 mM in 25 mM Tris-HCl (pH 7.6), 5 mM MgCl\textsubscript{2}, 5 mM DTT, 5 mM PEP, and 0.35\% decylPEG. The activity of the mutant was less than 0.1\% of the wild type activity at all mannitol concentrations. The same result was found for the mtl/mtl-P exchange kinetics measured in a reaction mixture containing
200 nM [3H]mannitol, 250 µM mtl-P, 25 mM Tris-HCl (pH 7.6), 5 mM MgCl₂, 5 mM DTT, and 0.35% decylPEG. Similar data was also obtained when these measurements were done with membrane vesicles containing EIIₘtl-G196D. Since the G196D mutation is situated in the mannitol-binding domain, the lack of activity might reflect a decreased affinity of the enzyme for mannitol. The dissociation constant of purified EIIₘtl-G196D for mannitol was examined using flow dialysis. In contrast with the situation with the purified wild type enzyme (Kₐ = 100 nM), no significant mannitol binding could be detected at mannitol concentrations up to 2 µM, indicating that the mutation lowers the affinity for mannitol by at least a factor of 20. The fact that no mannitol phosphorylation activity could be detected up to 16 mM indicates that there is no functional low-affinity binding either. This change in affinity for mannitol makes EIIₘtl-G196D a suitable candidate for complementation studies to examine the role of C domain interactions in the mechanism of transport and phosphorylation.

**Complementation Studies with the EIIₘtl-G196D Mutant.**

Previous studies with the EIIₘtl phosphorylation site mutants, H554A and C384S, have demonstrated that inactive proteins can complement each other, resulting in the restoration of in vitro PEP-dependent phosphorylation and mtl/mtl-P exchange activity as well as in vivo transport activity (van Weeghel et al., 1991; Weng et al., 1992). A high-affinity interaction with a Kₛ in the nanomolar range was also demonstrated to occur at the level of the membrane-bound C domain between purified EIIₘtl and purified IICₘtl. This interaction and the rates obtained are reproduced schematically from Boer et al. (1994) in panel I of Figure 5. To characterize this interaction further, complementation measurements were performed on the inactive mutant, EIIₘtl-G196D, and purified IICₘtl. The result of this complementation for the PEP-dependent phosphorylation reaction is shown in Figure 2A. When purified IICₘtl was added to the purified EIIₘtl-G196D, PEP-dependent phosphorylation activity could be measured. Increasing the IICₘtl concentration led to an increase in phosphorylation activity until saturation was observed. Since EIIₘtl-G196D can be complemented by an active, high-affinity binding site on IICₘtl, the B domain of EIIₘtl-G196D must be able to transfer its phosphoryl group to mannitol bound to IICₘtl. There is only one B domain present; IICₘtl lacks a B domain (panel II of Figure 5). To determine whether an additional cytoplasmic domain would have any effect on the above complementation, a similar experiment was done with purified EIIₘtl-C384S which is inactive because it lacks the second phosphorylation site (panel V of Figure 5). Again, PEP-dependent phosphorylation activity could be measured when EIIₘtl-G196D was titrated with EIIₘtl-C384S (Figure 2B). The concentration dependence of EIIₘtl-C384S was comparable to that observed for IICₘtl as was the maximal activity. Panels C and D of Figure 2 show the result for the mtl/mtl-P exchange reaction. Both IICₘtl and EIIₘtl-C384S can complement EIIₘtl-G196D in this reaction and show an enzyme concentration dependence similar to the phosphorylation kinetics. As usual, the specific activity of the exchange reaction is only a fraction of the phosphorylation activity (Table 1). These results show that active forms of EIIₘtl capable of in vitro PEP-dependent phosphorylation and mtl/mtl-P exchange can be created from mutants that, by themselves, are inactive due to nonfunctional B or C domains. However, a quantitative difference is evident when comparing the rates in panel II and V versus that in panel I; complementation with active enzyme results in much higher rates. This will be treated in the Discussion.

In order to determine whether in vivo mannitol transport could be restored and not just in vitro phosphorylation, a
Table 1: Complementation of EII<sup>mtl</sup>

<table>
<thead>
<tr>
<th>EII&lt;sup&gt;mtl&lt;/sup&gt; species</th>
<th>PEP-dependent phosphorylation&lt;sup&gt;a&lt;/sup&gt; [nmol of mtl-P min&lt;sup&gt;-1&lt;/sup&gt; (nmol of EII&lt;sup&gt;mtl&lt;/sup&gt;)&lt;sup&gt;-1&lt;/sup&gt;]</th>
<th>mtl/mtl-P exchange&lt;sup&gt;b&lt;/sup&gt; [nmol of mtl-P min&lt;sup&gt;-1&lt;/sup&gt; (nmol of EII&lt;sup&gt;mtl&lt;/sup&gt;)&lt;sup&gt;-1&lt;/sup&gt;]</th>
</tr>
</thead>
<tbody>
<tr>
<td>G196D</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>C384S</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>IIC&lt;sup&gt;mtl&lt;/sup&gt;</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>G196D/C384S</td>
<td>130</td>
<td>0.4</td>
</tr>
<tr>
<td>G196D + IIC&lt;sup&gt;mtl&lt;/sup&gt;</td>
<td>125</td>
<td>0.1</td>
</tr>
<tr>
<td>G196D + C384S</td>
<td>928</td>
<td>5.1</td>
</tr>
<tr>
<td>EII&lt;sup&gt;mtl&lt;/sup&gt; + IIC&lt;sup&gt;mtl&lt;/sup&gt;</td>
<td>1049</td>
<td>7</td>
</tr>
<tr>
<td>EII&lt;sup&gt;mtl&lt;/sup&gt; + G196D</td>
<td>900</td>
<td>6.3</td>
</tr>
<tr>
<td>EII&lt;sup&gt;mtl&lt;/sup&gt; + G196D/C384S</td>
<td>940</td>
<td>4.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> The data in rows 7–10 are specific activities for the wild type enzyme (EII<sup>mtl</sup>) at saturating concentrations of the second component.

<sup>b</sup> The PEP-dependent phosphorylation was measured at 30 °C with a 100 µL reaction mixture containing 25 mM Tris-HCl (pH 7.6), 5 mM MgCl<sub>2</sub>, 5 mM DTT, 5 mM PEP, 0.25% decylPEG, 60 µM [3H]<sup>-</sup>mannitol, 165 nM EII<sup>mtl</sup>, 17.6 µM HPr, and the purified EII<sup>mtl</sup> listed in the table. Four 20 µL aliquots were taken at various times and loaded onto Dowex AG1-X2 columns. A 10 µL aliquot was used to measure the total amount of radioactivity in the assay. The assay procedure has been described in detail by Robillard and Blaauw (1987). nd indicates not detectable and means that the phosphorylation activity was less than 1 nmol of mtl-P min<sup>-1</sup> (nmol of EII<sup>mtl</sup>)<sup>-1</sup>. c The data in rows 5 and 6 are the specific activities for the mutant enzyme (G196D) in the presence of saturating concentrations of the second component. d The mannitol/mannitol-P exchange was measured at 30 °C with a 100 µL reaction mixture containing 25 mM Tris-HCl (pH 7.6), 5 mM MgCl<sub>2</sub>, 5 mM DTT, 0.25% decylPEG, 250 µM mannitol 1-phosphate, 200 mM [3H]<sup>-</sup>mannitol, and membrane vesicles containing the mutant. The further assay procedure was identical to that described above. nd indicates not detectable and means that the exchange activity was less than 0.01 nmol of mtl-P min<sup>-1</sup> (nmol of EII<sup>mtl</sup>)<sup>-1</sup>.

The data in rows 5 and 6 are specific activities for the wild type enzyme (EII<sup>mtl</sup>) at saturating concentrations of the second component.

The PEP-dependent phosphorylation was measured at 30 °C with a 100 µL reaction mixture containing 25 mM Tris-HCl (pH 7.6), 5 mM MgCl<sub>2</sub>, 5 mM DTT, 5 mM PEP, 0.25% decylPEG, 60 µM [3H]<sup>-</sup>mannitol, 165 nM EII<sup>mtl</sup>, 17.6 µM HPr, and the purified EII<sup>mtl</sup> listed in the table. Four 20 µL aliquots were taken at various times and loaded onto Dowex AG1-X2 columns. A 10 µL aliquot was used to measure the total amount of radioactivity in the assay. The assay procedure has been described in detail by Robillard and Blaauw (1987). nd indicates not detectable and means that the phosphorylation activity was less than 1 nmol of mtl-P min<sup>-1</sup> (nmol of EII<sup>mtl</sup>)<sup>-1</sup>. c The data in rows 5 and 6 are the specific activities for the mutant enzyme (G196D) in the presence of saturating concentrations of the second component. d The mannitol/mannitol-P exchange was measured at 30 °C with a 100 µL reaction mixture containing 25 mM Tris-HCl (pH 7.6), 5 mM MgCl<sub>2</sub>, 5 mM DTT, 0.25% decylPEG, 250 µM mannitol 1-phosphate, 200 mM [3H]<sup>-</sup>mannitol, and membrane vesicles containing the mutant. The further assay procedure was identical to that described above. nd indicates not detectable and means that the exchange activity was less than 0.01 nmol of mtl-P min<sup>-1</sup> (nmol of EII<sup>mtl</sup>)<sup>-1</sup>.

A two-plasmid expression system was developed to allow the coexpression of EII<sup>mtl</sup> derivatives in E. coli. Strain LGS322 was transformed either with plasmid pJRDIIC expressing IIC<sup>mtl</sup> or with pMcG196D expressing EII<sup>mtl</sup>-G196D; it was also transformed with the two plasmids together. Cells grown at 37 °C expressing IIC<sup>mtl</sup> or EII<sup>mtl</sup>-G196D had a white phenotype on MacConkey plates containing 1% mannitol, while cells containing the two plasmids had a red/pink phenotype, indicative of mannitol fermentation. The uptake measurement was started by adding 10 µL of 35 µM [14C]<sup>-</sup>mannitol to these cells. After the appropriate time, the uptake was quenched by the rapid addition of 2 mL of ice-cold 50 mM KP<sub>i</sub> buffer (pH 7.5). The uptake measurement was started by adding 10 µL of 35 µM [14C]<sup>-</sup>mannitol to these cells. After the appropriate time, the uptake was quenched by the rapid addition of 2 mL of ice-cold 50 mM KP<sub>i</sub> buffer (pH 7.5) containing 1 mM HgCl<sub>2</sub>. The cell suspension was then rapidly filtered across Whatman GF/F filters, and the vessel and filter were washed twice with 2 mL of the quenching buffer. The cells on the filters were solubilized with scintillation liquid, and the amount of radioactivity in the cells was determined.

from the A domain to the B domain and then to mannitol on the C domain of a single EII<sup>mtl</sup> subunit. The following section addresses this issue for both the in vitro and in vivo processes using a double mutant.

Complementation of the EII<sup>mtl</sup>-G196D/C384S Mutant with EII<sup>mtl</sup>-H554A and Wild Type EII<sup>mtl</sup>. EII<sup>mtl</sup>-G196D/C384S possesses both an inactive C and B domain. To determine whether the A domain was still functional, it was complemented with EII<sup>mtl</sup>-H554A, a protein with an inactive A domain but active B and C domains. The PEP-dependent phosphorylation activity could be restored in a saturatable manner when increasing concentrations of EII<sup>mtl</sup>-G196D/C384S were combined with EII<sup>mtl</sup>-H554A just as shown in Figure 2 for the other mutants and domains. The occurrence of saturatable activity in the low nanomolar enzyme concentration range is evidence that the double mutant is still able to form functional heterodimers.

Complementation of the double mutant with wild type EII<sup>mtl</sup> enables us to address the issue of the role of the second subunit of the dimer in the phosphorylation and transport mechanism. If phosphoryl transfer across the dimer interface from the B domain on one subunit to the mannitol–C domain complex on the other subunit is an essential element of the
phosphorylation and transport mechanism, then heterodimers consisting of one wild type subunit and one BC domain double mutant should be inactive (panel VI of Figure 5). The experiments were carried out exactly as those in Figure 2 with a fixed concentration of wild type enzyme and increasing concentrations of the double mutant. The results of these experiments are given in Table 1 and panel VI of Figure 5. Addition of the double mutant to the wild type enzyme does not lead to a decrease in PEP-dependent phosphorylation or mtl/mtl-P exchange activity but, rather, to an increase just as seen by Boer et al. (1994) upon addition of IIICmt to wild type enzyme. These high rates indicate that, although phosphoryl group transfer across the subunit interface is possible, the preferred route lies within a single subunit. The question remains as to whether this is also true for mannitol transport. This was addressed by the coexpression of wild type EIImt and EIImt-G196D or EIImt-G196D/C384S.

E. coli strain JM101 which expresses wild type EIImt was grown on minimal medium containing glucose instead of mannitol to achieve a low expression level of wild type EIImt. Low wild type expression levels were chosen for two reasons. (1) Good initial uptake rates could be measured, and (2) the expression level of the wild type enzyme would be much lower than that of the mutant EIImt, resulting in a high percentage of heterodimers containing one wild type subunit and one mutant subunit. That linear initial rates of mannitol uptake can be measured in cells grown on glucose is clearly seen in Figure 4 (■). SDS–polyacrylamide gel electrophoresis (Figure 1, lane 2) also shows that only low amounts of EIImt are present in these cells. The same strain was transformed with plasmid pMaG196D or pMaG196D/C384S and grown under the same conditions until the cells reached an OD600 of 0.6, and then expression of plasmid-encoded protein was initiated by thermoduction. The SDS–polyacrylamide gel in Figure 1 shows that the level of chromosomally expressed wild-type enzyme (lane 2) is much lower than that of EIImt-G196D or EIImt-G196D/C384S derived from the overexpression plasmids (lanes 3 and 4, respectively). The initial uptake rates of the cells that overexpressed the mutant G196D or G196D/C384S, however, are similar to that of cells that only expressed wild type EIImt (Figure 4). The presence of an inactive mutant subunit does not affect the rate of uptake of mannitol, leading to the conclusion that mannitol uptake by the dimer occurs via a single subunit, without the necessary involvement of the other subunit of the dimer.

**DISCUSSION**

**Phosphoryl Group Transfer by EIImt.** The various heterodimer experiments presented above are summarized in Figure 5. We started with the observation, made previously by Boer et al. (1994), that the addition of purified IIICmt to purified EIImt (panel I) resulted in a stimulation of the specific phosphorylation and exchange activity with an apparent affinity of IIICmt for EIImt of less than 10 nM. This observation can be interpreted in two ways: (i) that heterodimers are being formed or (ii) that addition of increasing concentrations of IIICmt is shifting the equilibrium of a monomeric population of intact EIImt back into the homodimeric form. The experiment in panel II shows that the heterodimer explanation is the correct one. If IIICmt was only shifting a monomeric population of intact EIImt back to the homodimeric form, no activity would be observed in this panel since the intact enzyme is mutated in the C domain and is inactive. The experiment in panel I does not however discriminate between phosphoryl group transfer from the B to the C domain within one subunit versus transfer across the subunit interface because both subunits in the heterodimer possess active C domains. However, when IIICmt was added to EIImt-G196D, the PEP-dependent phosphorylation and mtl/mtl-P exchange activities of these inactive species were restored (panel II). The saturation kinetics that were observed are indicative of the formation of a complex between IIICmt and EIImt-G196D where the phosphoryl group must transfer across the subunit interface from the active B domain on EIImt-G196D to the mannitol bound to IIICmt. In this case, however, the rates of both phosphorylation and exchange are approximately 10-fold lower than in panel I. The lower rates are not due to the inactive nature of the C domain of EIImt-G196D, as such, because high rates are observed with a heterodimer of wild type EIImt and EIImt-G196D (panel III). Consequently, the lower rates in panel II must be due to the necessity of the phosphoryl group to transfer across the subunit interface.

The experiment in panel V indicates that the presence of the inactive B domain does not prevent such a transfer, but again, the rates are approximately 10-fold lower than when a wild type subunit is present. Here as well, the inactive nature of the B domain is not responsible for the lower rates...
Mannitol Transport by EII<sup>mtl</sup>. Coexpression of IIC<sup>mtl</sup> and EII<sup>mtl</sup>-G196D in E. coli indicates that these two enzymes also associate in their natural environment to form a minimal configuration in the membrane that is able to catalyze mannitol transport. When the double mutant, EII<sup>mtl</sup>G196D/C384S, is overexpressed relative to the uninduced wild type EII<sup>mtl</sup> in E. coli, heterodimers will also form and, due to the large excess of double mutant relative to wild type enzyme, we expect virtually all wild type EII<sup>mtl</sup> to be present in the heterodimer form with one active and one completely inactive subunit. Since the initial uptake rates are very similar in the presence and absence of the double mutant, we must conclude that a single subunit in the dimer is capable of catalyzing the whole transport reaction as well as the phosphorylation reaction. No negative dominance is observed.

The in vivo and in vitro complementation experiments show that EII<sup>mtl</sup> is a prime example of a protein in which facultative domain swapping occurs (Bennett et al., 1995), but there is a clear preference for the domains to function with their partners in the same subunit. If a single subunit is sufficient, even optimal, for catalyzing both phosphorylation and transport, what is the rationale for the EII<sup>mtl</sup> dimer? Two reasons which could apply to transport proteins in general are as follows.

(i) Subunit interactions may result in pore formation. The monomeric subunits of aquaporin form a tetramer with a central cavity, and within this cavity, each monomer forms a narrow pore (Engel et al., 1994). As in the case of EII<sup>mtl</sup>, Lindenthal and Schubert (1991) demonstrated that a monomeric form of the erythrocyte transporter transports anions but electron diffraction showed a dimer with one central cavity (Wang et al., 1993). The dimer interface of EII<sup>mtl</sup> may also form a pore which is involved in providing the transport route for the sugar across the membrane. Robillard and Beechey (1986), using membrane impermeable reagents, provided clear evidence for the occurrence of such a pore in the E. coli EII<sup>glc</sup> which, like EII<sup>mtl</sup>, is also known to be a dimer (Erni, 1986).

(ii) Cooperative binding site interactions may facilitate rapid transport. The erythrocyte glucose transporter is a tetramer with cooperative binding site interactions between the subunits. In this protein, subunit association is important for rapid substrate translocation (Zottola et al., 1995; Coderre et al., 1995). Pas et al. (1988) presented evidence for one high-affinity and one low-affinity mannitol binding site per dimer, implying cooperative subunit interactions which Lolkema et al. (1993) also suggested could be involved in rapid substrate translocation.

More mechanistic and structural work will be necessary to resolve this issue.

REFERENCES


