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Phosphorylation Site Mutants of the Mannitol Transport Protein Enzyme IImtl of Escherichia coli: Studies on the Interaction between the Mannitol Translocating C-Domain and the Phosphorylation Site on the Energy-Coupling B-Domain†

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ABSTRACT: Mannitol binding and translocation catalyzed by the C domain of the Escherichia coli mannitol transport protein enzyme IImtl is influenced by domain B. This interaction was studied by monitoring the effects of mutating the B domain phosphorylation site, C384, on the kinetics of mannitol binding to the C domain. The dissociation constants for mannitol to the C384 mutants in inside-out membrane vesicles varied from 45 nM for the wild-type enzyme to 306 nM for the mutants. The rate constants pertinent to the binding equilibrium were also altered by the mutations. The association rate of mannitol to the cytoplasmic binding site in the mutants was accelerated for all mutants. The exchange rate of bound mannitol on the wild-type enzyme was shown to be pH dependent with a \( pK_a \) of approximately 8 and increasing rates at higher pH. This rate was increased for all the mutants, but the \( pK_a \)s differed for the various mutants. The exchange rate for binding to the isolated IICmtl, however, was not pH dependent and exhibited a low rate. Exchange measured at 4°C showed that, of the two steps, binding and occlusion, involved in binding to wild-type EIImtl in inside-out vesicles, only one could be detected for the C384E and C384L mutants. This suggests that the mutations increased the rate of the occlusion step so that it was no longer separable from the initial binding step or that the mutations eliminated the occlusion step altogether. The change in the mannitol binding kinetics of the C domain indicates that the B and C domains of EIImtl influence each other’s conformation. Residues on either the B or C domain close to the second phosphorylation site, C384, play an important role in this process and may provide a mechanism by which the energy coupling within this enzyme takes place.

The mannitol transport system of Escherichia coli is a phosphoenolpyruvate-dependent group-translocation system able to couple the transport of mannitol to its phosphorylation (Lolkema & Robillard, 1992; Postma et al., 1993). The transport protein EIImtl \(^{1,2} \) consists of three domains, a membrane-bound C domain and two cytoplasmic domains, B and A. It is known that phosphorylated intermediates of the transport protein exist, His554 on the A domain and Cys384 on the B domain being the two residues which are phosphorylated (Pas & Robillard, 1988). The cysteine at position 384 is the second phosphorylation site; it receives its phosphoryl group via an internal transfer from the first site, His554, which is phosphorylated by a general cytoplasmic PTS protein, P-HPr. The phosphoryl group transfer from P-HPr to mannitol-P is shown in Scheme 1.

\[ (P)-HPr + \text{IICBA}^{\text{mtl}} \rightleftharpoons HPr + (P)-\text{IICBA}^{\text{mtl}} \]

\[ \text{mtl(out)} + (P)-\text{IICBA}^{\text{mtl}} \rightleftharpoons \text{mtl(P)(in)} + \text{IICBA}^{\text{mtl}} \]

It is known that the A, B, and C domains can exist as stable separate proteins (van Weeghel et al., 1991; Robillard et al., 1993; Boer et al., 1994). The IIA\(^{\text{mtl}} \) and IIB\(^{\text{mtl}} \) proteins can still be phosphorylated, the IIC\(^{\text{mtl}} \) protein still binds mannitol with an affinity that is comparable with that of wild-type enzyme, and in a reconstituted system consisting of these three proteins, mannitol can still be phosphorylated. Mechanistic studies on mannitol transport and phosphorylation have revealed that the coupling between these two processes is not absolute (Lolkema et al., 1991a). Evidence for facilitated diffusion and uncoupling of transport and phosphorylation has been found for several EIIs (Postma et al., 1976; Elferink et al., 1990; Ruijter et al., 1990, 1992). The membrane-bound C domain seems to be capable of translocating mannitol, and the mannitol phosphorylation step seems to be a separate process which can take place independently of the translocation step (Grisafi et al., 1989; Lolkema et al., 1990, 1991a). Even though these reactions
can be separated under artificial conditions, they appear to be coupled in the membrane. Kinetic data indicate that phosphorylation of the B domain Cys384 accelerates transport 1000-fold. Furthermore, alkylation of this residue alters the binding kinetics of mannitol to the C domain (Lolkema et al., 1991a,b). Currently, EIImtl, in both inside-out and right-side-out membrane vesicles, is able to bind mannitol (Lolkema et al., 1992). These studies, which were done with the unphosphorylated enzyme, revealed transitions between three binding states of EIImtl, binding to a cytoplasmic site and a periplasmic site and a third binding state, in which mannitol was occluded. This state might be an intermediate between the cytoplasmic- and periplasmic-bound states (Lolkema et al., 1992). These studies raise the question of how, mechanistically, a transport process carried out by one domain or protein can be catalyzed by the phosphorylation of a second domain or protein. Does the affinity for the substrate change or is there an effect on the steps involved in translocation of the substrate?

To study this mechanism, we made mutations in the B domain phosphorylation site (C384) which might mimic the effect of phosphorylation, and we measured the effects of these mutations on the kinetics of mannitol binding and exchange. This paper compares the binding kinetics of the mutants with both wild-type EIImtl (Lolkema et al., 1992) and IICmtl (Boer et al., 1994), which lacks the cytoplasmic A and B domains.

**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. Decyl-PEG was synthesized by B. Kwant at the Department of Chemistry, University of Groningen. [3-14C]mannitol (2.04 GBq/mmoll) was obtained from American. [3-14H(N)]mannitol (976.8 GBq/mmoll) was from NEN Research Products, and mannitol 1-phosphate was purchased from Sigma. MacConkey agar plates containing 1% D-mannitol; all the mutants at position 384 have a white, mannitol fermentation negative, phenotype on these plates.

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**Overexpression of the mutants was achieved by insertion of the A-P promoter with the c1857 A-repressor gene into the mutant plasmids. For this purpose an EcoRI-SaI fragment containing the P promoter and repressor gene was excised from pJR187 and ligated into the corresponding restriction sites in the plasmid. This strategy for obtaining overexpression was identical to that published previously for the wild-type enzyme and IICmtl (van Weeghel et al., 1990; Boer et al., 1994). The resulting overexpression vector contained the mutant enzyme behind a tandem P, P promoter. The plasmids of the C384 mutants were designated pMaICPl, pMaC384D, pMaC384K, pMaC384H, pMaC384E, and pMaC384G.

The entire mtlA gene in each mutant plasmid was sequenced by the method of Sanger et al. (1977) and was identical to the previously published sequence of the gene (Lee & Saier, 1983), except for the mutation introduced.

**Expression and Preparation of Inside-Out Membrane Vesicles.** E. coli strain LGS322 was used for expression of EIImtl, IICmtl, and the mutants of EIImtl. The plasmid-containing cells were grown in a 1-L culture of LB medium at 37 °C until an OD600 of 1 was reached. If an overexpression plasmid was used, the culture was grown at 30 °C until an OD600 of 0.7 and then raised to 42 °C and grown for another 2 h. After the cells were harvested by centrifugation for 10 min at 5000g and 4 °C, they were washed with 25 mM Tris-HCl, pH 7.5, and centrifuged. The yield for both procedures was approximately 5 g of cells, wet weight. The washed cells were kept on ice and used to produce inside-out membrane vesicles as described by Lolkema and Robillard (1990). The membrane vesicles were suspended in 15 mL of 25 mM Tris-HCl, pH 7.5, and 1 mM DTT and

| Table 1: Primers Used To Construct the Cys384 Mutants |
| Mutant  | Primer 5'---3' |
| C384S   | CGCGGCTCGGCAAGCAGTGG |
| C384D   | CGCGGCTCGGCAAGCAGTGG |
| C384L   | CGCGGCTCGGCAAGCAGTGG |
| C384K   | CGCGGCTCGGCAAGCAGTGG |
| C384H   | CGCGGCTCGGCAAGCAGTGG |
| C384E   | CGCGGCTCGGCAAGCAGTGG |
| C384G   | CGCGGCTCGGCAAGCAGTGG |

Previously (Boer et al., 1994). Plasmid pJR187 was described previously (Davison et al., 1987). All E. coli strains were grown on LB medium (10 g of bactotryptone, 5 g of yeast extract, 10 g of NaCl per liter) containing 25 μg/mL chloramphenicol for strain CJ236 or 100 μg/mL ampicillin for all other plasmids used.
stored in small quantities in liquid nitrogen. The membrane vesicles were thawed only once and kept on ice until used. Material remaining at the end of the day was discarded.

**PEP-Dependent Mannitol Phosphorylation and Mannitol/ Mannitol-P Exchange Assays.** The PEP-dependent phosphorylation kinetics of EIIm and the mutant proteins were measured in 25 mM Tris-HCl, pH 7.6, 5 mM MgCl₂, 5 mM DTT, 5 mM PEP, and 0.25% decyl-PEG at 30 °C. The concentration of enzyme I, HPr, and labeled mannitol depended on the experiment. Details are given in the figure legends and the text. The volume of the assay mixture was 100 μL. Four 20-μL samples were taken at various times and loaded onto Dowex AG1-X2 columns. A 10-μL 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-P exchange assays were done at 30 °C in 25 mM Tris-HCl, pH 7.6, 5 mM MgCl₂, 5 mM DTT, 0.25% decyl-PEG, and a given concentration mannitol 1-phosphate. The exchange reaction was started with [³H]-mannitol. The assay volume and the sample size were the same as for PEP-dependent phosphorylation assays. The assay procedure has been described by Lolkema and Robillard (1990).

**Flow Dialysis.** The flow dialysis procedure of Lolkema et al. (1990, 1991b, 1992) was used for measuring the affinity of the enzyme for mannitol and for measuring the kinetics of mannitol binding. The flow dialysis system and dialysis buffer were thermostatted with a water bath at 25 °C. The experiments at 4 °C were done in a cold room. The flow dialysis cell was connected to a fraction collector with a drop counter; the time per fraction was constant over a very long period. The flow rate of the buffer through the lower compartment of the dialysis system was 0.5 mL/min, resulting in a half-time for response of the system of 6 s at 25 °C and 10 s at 4 °C. The data were corrected for the response of the system by subtracting the half-time of the response from the half-time found in the exchange experiment (Lolkema et al., 1990). The buffer conditions were thermostatted with a water bath at 25 °C and 10 s at 4 °C. The data were corrected for the response of the system by subtracting the half-time of the response from the half-time found in the exchange experiment (Lolkema et al., 1990).

**Protein Determinations.** Protein concentrations in the preparations were determined by the method of Bradford (1976) with BSA as the standard. The wild-type and mutant EIIm concentrations in membrane vesicles were quantitated by flow dialysis as the number of mannitol binding sites extrapolated from a Scatchard plot of mannitol binding. The expression of the mutant proteins was also monitored with western blotting using polyclonal antibodies raised against purified IIBim. The western blot procedure for the detection of EIIm was described by Pas et al. (1987).

**RESULTS**

**PEP-Dependent Mannitol Phosphorylation and Mannitol/ Mannitol-P Exchange Kinetics of the Mutants.** Mutation of the second phosphorylation site, Cys384, leads, in most cases, to a complete loss of both the PEP-dependent phosphorylation activity and the mannitol/mannitol-P exchange activity as is to be expected if the phosphocysteine is an intermediate during catalysis (Pas & Robillard, 1988; van Weeghel et al., 1991; Weng et al., 1992). The activities reported in Table 2 have been corrected for the expression levels of EIIm and its mutants, because western blots showed that the expression levels differed when equal amounts of membrane protein were applied on the gels. For this reason the amount of mannitol binding sites was taken as the measure of the concentration of the mutant protein in the membrane.

None of the Cys384 mutants showed any PEP-dependent phosphorylation activity. A similar result was obtained for the mannitol/mannitol-P exchange activity except for the C384D mutant that retained 1.2% of the wild-type exchange activity. No mannitol/mannitol-P exchange could be measured with the C384E mutant. As a control experiment, NEM alkylation was used to inactivate cysteine 384, leading to an almost complete loss of activity (less than 1% activity after inhibition with NEM). The C384D enzyme, however, is not sensitive to NEM inactivation and retained the same level of activity as the untreated protein. The C384D mannitol/mannitol-P exchange activity is much lower than that found by Weng et al. (1992). The reason for the difference is not clear.

**Affinity of the Mutants for Mannitol.** The dissociation constants for mannitol were measured to determine in what way mutations at the phosphorylation site in the B domain would influence mannitol binding to the C domain. Table 3 shows that the affinity of the plasmid-encoded wild-type EIIm is in good agreement with that reported for enzyme from *E. coli* strain ML308-225, our previous source of EIIm; the affinity in detergent is somewhat lower than in membrane

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**Table 2: PEP-Dependent Phosphorylation and Mannitol/ Mannitol-P Exchange Activities of the Mutants**

<table>
<thead>
<tr>
<th>mutant</th>
<th>PEP-dependent phosphorylation activity (%) of WT</th>
<th>mannitol/mannitol-P exchange activity (%) of WT</th>
<th>NEM inhibition of the exchange (% residual activity)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>100b</td>
<td>100c</td>
<td>0.5</td>
</tr>
<tr>
<td>C384D</td>
<td>0c</td>
<td>1.2</td>
<td>100</td>
</tr>
<tr>
<td>C384E</td>
<td>0c</td>
<td>0c</td>
<td>0c</td>
</tr>
<tr>
<td>C384S,L,K,H,E,G</td>
<td>0c</td>
<td>0c</td>
<td>0c</td>
</tr>
</tbody>
</table>

Notes:

- 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 MgCl₂, 5 mM DTT, 5 mM PEP, 0.25% decyl-PEG, 3.5 μM [³H]mannitol, 100 nM EL, 17.6 μM HPr, and membrane vesicles containing the mutant. 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).

- The 100% PEP-dependent phosphorylation activity corresponds with an activity of 376 nmol of mtl-P/(min·mmol of EIIm). The 0% corresponds with a background activity of 0.4%, which is also present under the same conditions in membranes derived from *E. coli* strain LGS322 cells grown without the plasmids.

- 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 MgCl₂, 5 mM DTT, 0.25% decyl-PEG, 250 μM mannitol 1-phosphate, and 200 nM [³H]mannitol and membrane vesicles containing the mutant. The further assay procedure was identical to that described above.

- The 100% exchange activity corresponds with an activity of 0.8 nmol of mtl-P/(min·mmol of EIIm).

- Under the experimental conditions no background activity could be detected in membranes of *E. coli* strain LGS322 grown without the plasmids.
vesicles (Lolkema et al., 1990, 1991b). IIC\textsuperscript{end} still binds mannitol with a high affinity both in vesicles and in detergent, as do all Cys384 phosphorylation site mutants; however, there are quantitative differences. The affinity in inverted membrane vesicles ranges from 45 nM for the wild-type enzyme to 306 nM for the C384G mutant. With some of the mutants, solubilization hardly affects the dissociation constant, whereas, with C384H and C384E, there is a significant increase in affinity upon solubilization.

Table 3: Affinities for Mannitol in the Presence and Absence of Decyl-PEG\textsuperscript{a}

<table>
<thead>
<tr>
<th>enzyme</th>
<th>enzyme in vesicles</th>
<th>enzyme solubilized</th>
<th>$K_d$ (nM), 25 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>EIIf\textsuperscript{end}</td>
<td>45</td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>IIC\textsuperscript{end}</td>
<td>139</td>
<td>142</td>
<td></td>
</tr>
<tr>
<td>C384D</td>
<td>96</td>
<td>105</td>
<td></td>
</tr>
<tr>
<td>C384S</td>
<td>107</td>
<td>104</td>
<td></td>
</tr>
<tr>
<td>C384L</td>
<td>151</td>
<td>111</td>
<td></td>
</tr>
<tr>
<td>C384K</td>
<td>168</td>
<td>128</td>
<td></td>
</tr>
<tr>
<td>C384H</td>
<td>232</td>
<td>153</td>
<td></td>
</tr>
<tr>
<td>C384E</td>
<td>263</td>
<td>113</td>
<td></td>
</tr>
<tr>
<td>C384G</td>
<td>306</td>
<td>288</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} The binding of $[^3H]$mannitol to these enzymes was determined by adding 50, 99, 196, 385, and 566 nM $[^3H]$mannitol to the upper compartment of the flow dialysis cell containing 400 $\mu$L of 1.2–3.6 mg/mL vesicles in 25 mM Tris-HCl, pH 7.5, 5 mM MgCl\textsubscript{2}, and 5 mM DTT in the presence or absence of 0.5% decyl-PEG. The temperature was 25 °C. The amount of free mannitol in the upper compartment was measured at the indicated mannitol concentrations, by collecting samples of the flow and determining the amount of labeled mannitol in these samples. The total amount of mannitol in the vesicle suspension in the upper compartment was monitored by adding 50, 99, 196, 385, and 566 nM $[^3H]$mannitol to the upper compartment of the dialysis cell. This solution contained 25 mM Tris-HCl, pH 7.5, 5 mM MgCl\textsubscript{2}, 5 mM DTT, and 1.2–3.6 mg/mL iso membrane vesicles. The amount of free mannitol in the upper compartment was measured by collecting samples of the flow through the lower compartment with a fraction collector and determining the amount of $[^3H]$mannitol in these samples. The total amount of mannitol in the vesicle suspension in the upper compartment was then plotted in Scatchard plots.

Figure 1: Kinetics of binding of mannitol to EIIf\textsuperscript{end}, IIC\textsuperscript{end}, and the C384E, C384L, and C384K mutants. The rate of binding of mannitol to the enzymes was monitored by adding, at $t = 0$, 4 $\mu$L of 10 $\mu$M $[^3H]$mannitol to 400 $\mu$L of the vesicle suspension in the upper compartment of the flow dialysis cell. This solution contained 25 mM Tris-HCl, pH 7.5, 5 mM MgCl\textsubscript{2}, 5 mM DTT, and 1.2–3.6 mg/mL iso membrane vesicles. The amount of free mannitol in the upper compartment was measured by collecting samples of the flow through the lower compartment with a fraction collector and determining the amount of $[^3H]$mannitol in these samples. The total amount of mannitol in the vesicle suspension in the upper compartment was then plotted in Scatchard plots.

Influence of Cys384 Mutations on the Kinetics of Mannitol Binding and Exchange. The kinetics of mannitol binding to wild-type EIIf\textsuperscript{end} has been well characterized (Lolkema et al., 1992). The process was slow for the enzyme in iso vesicles compared to the enzyme in iso vesicles, even though the affinity for the substrate did not differ much for both orientations. The slow binding was detected by following the equilibration of the binding of mannitol to EIIf\textsuperscript{end} with flow dialysis. Panel A of Figure 1 shows the binding of mannitol to EIIf\textsuperscript{end} (\textbullet) in iso vesicles. At $t = 0$ 100 nM $[^3H]$mannitol was added to the membrane suspension, and the amount of free mannitol was monitored. The overshoot is the result of the fast response of the flow dialysis system in combination with a slow binding of mannitol to EIIf\textsuperscript{end} in the upper compartment of the dialysis cell (Lolkema et al., 1992). Mannitol binding to IIC\textsuperscript{end} in iso vesicles gives a similar result (Panel A, ◯). The exact rate of the binding process is difficult to quantify since the curve is a combination of the response of the flow dialysis system and the equilibration process. The overshoot found for EIIf\textsuperscript{end} and IIC\textsuperscript{end} has almost disappeared in the case of the C384E (*) and C384L (○) mutants and was not detectable for the C384K (△) mutant, indicating that the rate of mannitol binding to the enzyme was increased by these mutations (panel B). The rate of binding could be slowed down by repeating the experiments at 4 °C (panel C). The binding levels did not change substantially, but the overshoot which was detectable for the EIIf\textsuperscript{end} at 25 °C became visible for the mutants at 4 °C.
Domain Interactions in Mannitol Transport Protein

After 3 min of incubation at 25 °C 400 μL of this sample was loaded into the upper compartment of the flow dialysis cell and 450-μL sample containing 25 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 5 mM DTT, and iso membrane vesicles (1.2 mg/mL for EII₈ and 3.6 mg/mL for the C384E mutant) derived from non-overproducers. After 3 min of incubation at 25 °C 400 μL of this sample was loaded into the upper compartment of the flow dialysis cell operating at the same temperature, and the amount of bound and free mannitol was determined by collecting five samples. At the time indicated with an arrow, 4 μL of 10 mM unlabeled mannitol was added to the upper compartment. The exchange of bound [³H]mannitol with unlabeled mannitol was followed by collecting samples of the flow with a fraction collector and determining the amount of [³H]mannitol in these samples. The half-time of the instrument response was 6 s under these conditions. The figure shows an example of this experiment with EII₈ (●) and the C384E mutant (○).

The kinetics of dissociation can also be measured by flow dialysis (Lolkema et al., 1990, 1992a). Figure 2 shows the exchange of [³H]mannitol bound to EII₈ (●) and the C384E mutant (○) with an excess of unlabeled mannitol. The first five points indicate the amount of [³H]mannitol that is still free after equilibrium has been reached (62 nM bound mannitol of the 100 nM added). At the point indicated with the arrow, a 1000-fold excess of nonradioactive mannitol was added to the sample, and the rate of displacement of the bound [³H]mannitol was followed. The rate of displacement is clearly increased as a result of the C384E mutation. Analysis of the residual binding as a function of time reveals half-times of 43 and 6 s for the wild-type and C384E, respectively. The residual binding of [³H]mannitol to EII₈ and IIC₈ after addition of excess unlabeled mannitol was analyzed at different pH values (Figure 3A,B). The drawn lines are the fit to the experimental data plotted in the figure assuming a single exponential (Lolkema et al., 1991b). The dissociation rate of mannitol bound to the intact enzyme increased dramatically with pH (Figure 3A). This same pH dependence is not seen with isolated IIC₈ (Figure 3B). Table 4 lists the dissociation rates obtained at pH 7.5 for EII₈ and IIC₈ calculated from the fits in Figure 3 together with those for the mutant proteins. The rates measured for the wild-type enzyme and IIC₈ are comparable and in good agreement with those reported previously (Lolkema et al., 1990). Mutating the C384 phosphorylation site leads to an increased exchange rate in all cases. To confirm these changes in the rates, some of the measurements were repeated at 4 °C, and the same trend was observed as at 25 °C.

**pH Dependence of the Mutant Protein Exchange Kinetics.** From the kinetics of binding and exchange we must conclude that mutations at the C384 phosphorylation site affect the substrate binding kinetics of EII₈. All amino acid replacements lead to the same effect, an increase of the binding rates and the rates of exchange of bound mannitol. The acceleration of these processes could be due to a direct interaction between the mutated amino acid side chain and the mannitol binding site or it could arise indirectly as a result of altered interactions in the B domain which, in turn, affect B-C domain interactions. pH-dependent kinetics have been employed to discriminate between these two options. The pH dependence of the exchange rate shows a pKₐ of 7.8-8 for EII₈ (Figure 4, ●); at higher pH the exchange process is accelerated. By contrast, the exchange rate for IIC₈ is slow and independent of pH. These data suggest that the pH effect is caused by the cytoplasmic domains of EII₈. To see whether this effect is due to the active site cysteine itself or some other residue, the measurements were repeated with various C384 mutants. A pH dependence similar to that of native EII₈ was found for C384S, although the curve is shifted to lower pH, with a calculated pKₐ of 7.2. This result indicates that the pH dependence of mannitol binding is not due only to the active site cysteine but to other residues on the B domain which sense the cysteine or its replacement.

To measure whether the charge of the side chain affects the binding and exchange kinetics, the lysine and glutamate mutants were studied. The C384E mutation leads to an absolute rate increase over the whole pH range, but the pKₐ is not substantially different from that of the C384S mutant (pKₐ = 7.0). The lysine mutant shows a further rate increase at higher pH, with a calculated pKₐ of 7.2. This result indicates that the pH dependence of mannitol binding is not due only to the active site cysteine but to other residues on the B domain which sense the cysteine or its replacement.

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**Table 4:** Exchange of Bound [³H]Mannitol and Free Unlabeled Mannitol

<table>
<thead>
<tr>
<th>enzyme</th>
<th>25 °C (s)</th>
<th>4 °C (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EII₈</td>
<td>43</td>
<td>190</td>
</tr>
<tr>
<td>IIC₈</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td>C384D</td>
<td>11</td>
<td>53</td>
</tr>
<tr>
<td>C384S</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>C384L</td>
<td>7</td>
<td>35</td>
</tr>
<tr>
<td>C384K</td>
<td>5</td>
<td>26</td>
</tr>
<tr>
<td>C384H</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>C384E</td>
<td>6</td>
<td>49</td>
</tr>
<tr>
<td>C384G</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

*The exchange rates at 25 and 4 °C were measured as described for the data in Figure 2. The half-times of exchange were corrected for the response time of the system at that temperature: t₁/₂ = 6 s at 25 °C and t₁/₂ = 10 s at 4 °C.*
FIGURE 3: pH dependence of the exchange rate of EIImtl and IICmtl. These exchange experiments were performed as explained in the legend to Figure 2, except that 25 mM Bis/Tris-HCl buffers were used for the pH range 5.5–7.0 and 25 mM Tris-HCl for the pH range 7.5–9. The dialysis buffer has the same pH as the buffer in the upper compartment. The experiments were done with iso membrane vesicles derived from *E. coli* strain LGS322 transformed with the overproducing plasmids pMamtlAP, and pMaICP, for production of EIImtl and IICmtl, respectively. As a result it was possible to dilute the vesicles 18 times in a buffer of the desired pH, containing MgCl₂ and DTT, as described by Reenstra et al. (1980). After dilution, the vesicles were incubated on ice for 45 min to allow the internal and external pH to equilibrate. The sample was incubated at 25 °C for 5 min before the flow dialysis exchange measurements were begun. The measurements were done at 25 °C. The membrane protein concentration in the experiment was 0.2–0.4 mg/mL. The residual binding after addition of excess unlabeled mannitol is plotted versus time. The lines are a fit of the data points to a single exponential. Panels: (A) exchange measured for the wild-type EIImtl between pH 6 and 9; (B) exchange measured for IICmtl between pH 6 and 9. The symbols indicate pH 6.0 (○), pH 7.0 (●), pH 7.5 (▲), pH 8.0 (*), and pH 9.0 (○).

EIImtl in iso vesicles.

Scheme 2

\[ \text{EI}_{\text{cyt}} + \text{mtl} \rightleftharpoons \text{EI}_{\text{cyt}}\text{mtl} \rightleftharpoons \text{EI}_{\text{equ}}\text{mtl} \]

Mannitol binds first to the exposed site at the cytoplasmic (outside) side of the iso vesicles, EIImtl, and then the enzyme isomerizes to give an occluded state, EIImtl. One of the key experiments leading to this conclusion was repeated with wild-type EIImtl and, for comparison, with mutants C384E and C384L (Figure 5). The exchange of bound [³H]mannitol with an excess of unlabeled mannitol was measured at 4 °C using iso vesicles. The exchange process was followed at different stages during the equilibration of the [³H]mannitol binding. A large excess of unlabeled mannitol was added at the top of the overshoot, before the binding of 100 nM [³H]mannitol had equilibrated (compare Figure 1A, ○, and Figure 5A, ○). The experiment was repeated after incubation for 90 min with 100 nM [³H]mannitol, resulting in a complete equilibration of the binding (Figure 5A, ●). The residual binding is analyzed in panel D. In the latter experiment the displacement is much slower after equilibration of the binding than observed in the initial stages. These results are similar to what has been published before (Lolkema et al., 1992). The faster displacement represents dissociation from state EIImtl; the slower, from EIImtl (Scheme 2).

The experiment was repeated with mutants C384E and C384L to measure whether there was an effect of these mutations on the two steps involved in binding to the cytoplasmic site (Figure 5B,C,E,F). In contrast with the wild type in panel A, very little difference in the rates is observed with the mutants. The slow component, present in the wild-type enzyme, is no longer observed with these mutants of C384.

In terms of a two-step binding model the mutant data suggest that either the transition to the EIImtl state no longer occurs or that it is too fast in the mutants to be detected as a separate transition by this method.

DISCUSSION

The carbohydrate-specific enzyme IIs of phosphotransferase systems are multidomain proteins, most of them consisting of a membrane-bound domain and one or two cytoplasmic domains. Each cytoplasmic domain contains a phosphorylation site, a phosphohistidine intermediate in domain A and a phosphocysteine in domain B, except in the case of EIIcm (Erni et al., 1989). A major question is how this type of transport protein is capable of using the energy involved in protein phosphorylation for translocation and phosphorylation of their carbohydrate substrates.

The following observations indicate that the coupling between transport and phosphorylation is not absolute:

(i) EIIs are known to catalyze facilitated diffusion under certain conditions (Postma et al., 1976; Elfertnk et al., 1990).

(ii) Mutants of EIImtl have been obtained which catalyze transport in the absence of phosphorylation at rates high enough to allow cell growth (Ruijter et al., 1990, 1992).

(iii) Mutants of EIImtl have been obtained which catalyze phosphorylation without transport (Manayan et al., 1988; Buhr et al., 1992).
mutations and the detergent might interfere with the interaction between domains B and C, as was originally suggested for C384S by Lolkema et al. (1991b). The C384G mutant seems to be an exception to this; no effect of decyl-PEG could be measured, suggesting that the glycine substitution leads to larger changes which may involve other portions of the B domain and other B–C domain interactions. The reported dissociation constants are comparable with the values that were found by Weng and Jacobson (1993) for the mutants C384D and C384H.

**Binding Kinetics.** Lolkema et al. (1992) showed that the association of mannitol to the cytoplasmic site of the wild-type enzyme in iso vesicles was slow. A kinetic determination of binding to the enzyme in the opposite orientation was not possible; the equilibration was too fast to be detected with flow dialysis. The binding of mannitol to IIC\textsuperscript{mut}, which lacks the cytoplasmic domains, also slowly equilibrates comparable with the wild-type enzyme. The binding rate, however, was increased by mutations at C384; at 25 °C no overshoot was detectable. Lowering the temperature to 4 °C slowed down the binding in the mutants so that the overshoot was visible again. Clearly, mutations at C384 in the B domain affect the binding kinetics of mannitol binding to the C domain.

These domain interactions were further investigated by measuring the exchange rates for bound mannitol for the enzyme in iso vesicles. Chemical modification of C384 with NEM and Hg\textsuperscript{2+} affected the exchange process (Lolkema et al., 1991b). Mutations at position 384 seem to have the same effect; exchange is accelerated, although the absolute effect of the various mutations differs.

A clear pH dependence of the exchange rate has been found for the wild-type enzyme; at higher pH the process is accelerated. The pH dependence was still observed in the mutant enzymes, suggesting that residues other than the active site cysteine are responsible for the pH dependence of the mannitol binding. Since IIC\textsuperscript{mut} is insensitive to pH, the residues responsible for the pH dependence are most likely situated in the B domain and positioned close to the active site cysteine. The pK\textsubscript{s} for approximately 7 for the various mutants makes histidine a likely suspect. The B domain possesses four histidines distributed evenly through its structure. Further site-directed mutagenesis will be necessary to determine whether one of them is responsible for the pH dependence reported above. The glutamate mutant shows an absolute increase of the rate over the whole pH range, but the pK\textsubscript{s} is not changed much compared with mutant C384S. The exchange rate of the C384K mutant is increased over the whole pH range. The fact that oppositely charged residues, glutamate and lysine, both result in a substantial exchange rate enhancement and that the glutamate and serine mutants both show the same pK\textsubscript{s} indicates that there is no simple relationship between the rate changes, pK\textsubscript{s} changes, and charged state. One might expect such a relationship if the changes were due to a localized electrostatic interaction between the residue at position 384 and some other residue in the B domain. Most probably the kinetic changes reflect broader changes in the B domain structure and its interaction with the C domain.

The mechanism for binding of mannitol from the cytoplasmic side to wild-type EIImut involves two steps, as shown in Scheme 2 (Lolkema et al., 1992).

Our ability to kinetically discriminate between these steps in the wild-type enzyme almost completely disappears in the mutants C384E and C384L. The same was observed with NEM-alkylated enzyme (not shown). When the exchange of bound mannitol was measured before and after the binding had reached equilibrium, there was a much smaller difference.
Figure 5: Exchange of bound mannitol before and after equilibration of the binding. A 850-μL sample containing 25 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 5 mM DTT, and iso membrane vesicles was loaded into the upper compartment of the flow dialysis cell operating at 4 °C in a cold room. For the experiment with EII₅₉₆ vesicles derived from the overproducer were used (0.4 mg/mL membrane protein). For the C384E and C384L mutant experiments vesicles derived from a non-overproducer were used (C384E, 3.6 mg/mL membrane protein; C384L, 2.7 mg/mL membrane protein). At t = 0, 5.5 μL of 10 μM [³H]mannitol was added to the upper compartment, and the binding was monitored. After the sample was properly mixed, 450 μL was taken out of the upper compartment and stored at 4 °C for 90 min. To the sample that remained in the flow dialysis cell was added 4 μL of 10 mM unlabeled mannitol at the top of the overshoot indicated with an arrow (open symbols). The exchange of bound [³H]mannitol with unlabeled mannitol was followed by collecting samples of the flow with a fraction collector and determining the amount of [³H]mannitol in these samples. After 90 min 400 μL of the sample that was stored at 4 °C was reloaded into the flow dialysis cell, and five fractions were collected to measure the amount of mannitol that is free in this sample. Then 4 μL of 10 mM unlabeled mannitol was added, and the exchange of bound [³H]mannitol with unlabeled mannitol was measured (filled symbols). To determine the end level, 0.5% decyl-PEG was added, which disrupts the vesicles and causes a rapid exchange of the [³H]mannitol that is still bound. Panels A–C: the experiment described above with wild-type EII₅₉₆, the C384E mutant, and the C384L mutant, respectively. Panels D–F: residual binding versus time plots of the experiments described under panels A–C. The half-times of exchange are (D) wild-type EII₅₉₆, t₁/₂ = 190 s (●) and t₁/₂ = 110 s (○). (E) mutant C384E, t₁/₂ = 49 s (●) and t₁/₂ = 39 s (○), and (F) mutant C384L, t₁/₂ = 35 s (▲) and t₁/₂ = 23 s (△).

In the measured rates than in the wild-type EII₅₉₆. This indicates that the isomerization to the occluded state is affected by the mutations. The fast process, before equilibration in the wild-type enzyme, has a rate very similar to that found for the mutants before equilibration. But the slow process seen in the wild-type enzyme after equilibration is not observed in either mutant. Either the rate of conversion to the occluded state is substantially enhanced in the mutants such that it is no longer separable from the initial binding reaction or the conversion to the occluded state is completely inhibited in the mutants such that one only observes the initial binding reaction.
The C384D and C384E mutations of the phosphorylation site have different effects on the mannitol/mannitol-P exchange reaction. As found previously by Weng et al. (1992) the C384D mutant is active only in mannitol/mannitol-P exchange; however, we observe a much lower activity than reported by Weng et al. (1992). The differences may be due to an underestimate by Weng et al. of their EII<sup>cd</sup> content in the membrane. In our case, the content has been quantitated by mannitol binding, enabling us to report specific activities. Further study will be necessary to determine whether the exchange activity is due to a phosphoaspartate intermediate. This would be especially interesting since the mutant is not capable of receiving a phosphoryl group from His554 at the first phosphorylation site of domain A. Mutant C384E was not active in either reaction, indicating that the length of the side chain is important for the activity of these mutants with a carboxyl group-containing side chain. This finding might be interesting for other proteins in which phosphocysteine and phosphoaspartate intermediates are known.

**SUMMARY**

These binding studies with site-directed mutants show that there is an interaction between domains C and B of EII<sup>cd</sup>, which is affected by solubilization in detergent. The nature of the side chain at the phosphorylation site is important for this interaction. Mutations at this position affect residues on either the B or C domain and thereby the binding of mannitol to domain C. The two-step binding mechanism for EII<sup>cd</sup> is explained by Lolkema et al. (1992) as an isomerization between two conformational states of the enzyme. Mutations in the B domain phosphorylation site influence these binding states, which are probably different states of the C domain. Affecting residues in domain B, which are important for B–C domain interaction, by phosphorylation could result in a conformational change within domain C. This might be a mechanism by which the enzyme uses phosphorylation at cysteine 384 to catalyze the translocation of mannitol by the C domain.

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**REFERENCES**


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