Localization of the Substrate-binding Site in the Homodimeric Mannitol Transporter, EII\textsuperscript{mtl}, of \textit{Escherichia coli}\textsuperscript{*}\textsuperscript{S}

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The mannitol transporter from \textit{Escherichia coli}, EII\textsuperscript{mtl}, belongs to a class of membrane proteins coupling the transport of substrates with their chemical modification. EII\textsuperscript{mtl} is functional as a homodimer, and it harbors one high affinity mannitol-binding site in the membrane-embedded C domain (IIC\textsuperscript{mtl}). To localize this binding site, 19 single Trp-containing mutants of EII\textsuperscript{mtl} were biosynthetically labeled with 5-fluorotryptophan (5-FTrp) and mixed with azi-mannitol, a substrate analog acting as a Förster resonance energy transfer (FRET) acceptor. Typically, for mutants showing FRET, only one 5-FTrp was involved, whereas the 5-FTrp from the other monomer was too distant. This proves that the mannitol-binding site is asymmetrically positioned in dimeric IIC\textsuperscript{mtl}. Combined with the available two-dimensional projection maps of IIC\textsuperscript{mtl}, it is concluded that a second resting binding site is present in this transporter. Active transport of mannitol only takes place when EII\textsuperscript{mtl} becomes phosphorylated at Cys\textsuperscript{384} in the cytoplasmic B domain. Stably phosphorylated EII\textsuperscript{mtl} mutants were constructed, and FRET experiments showed that the position of mannitol in IIC\textsuperscript{mtl} remains the same. We conclude that during the transport cycle, the phosphorylated B domain has to move to the mannitol-binding site, located in the middle of the membrane, to phosphorylate mannitol.

Membrane-embedded transport proteins can be divided in three different classes based on their energy-coupling mechanism (1): (i) primary transport systems, which comprise transporters that use chemical energy or light to actively transport a specific solute over the membrane; (ii) secondary transporters, which are transporters in which the uphill transport of a solute is coupled to the downhill transport of a chemical compound along a gradient; and (iii) group translocation systems, which couple active transport with the chemical modification of the solute. The Enzyme II sugar transporters of the phosphoenolpyruvate-dependent group translocation system (PTS)\textsuperscript{2} (2, 3) are the only known transporters belonging to the third class. These transporters phosphorylate their sugar during transport over the membrane. The \textit{Escherichia coli} PTS transporters specific for glucose, β-glucosides, mannitol, and mannose are the best characterized members (4–9). Enzyme II proteins consist of two cytoplasmic domains, IIA and IIB, involved in phosphoryl group transfer, and a membrane-spanning IIC domain, showing the sugar specificity (10). In some Enzyme II proteins, like the mannose transporter, a second membrane-spanning domain, IID, is present. Depending on its sugar specificity, Enzyme II occurs as separate domains or as fused constructs between two or three domains (11). In EII\textsuperscript{mtl}, the mannitol-specific transporting and phosphorylating enzyme from \textit{E. coli}, all three domains IIA\textsuperscript{mtl} (12), IIB\textsuperscript{mtl} (13), and IIC\textsuperscript{mtl} (membrane-embedded domain of EII\textsuperscript{mtl}) are covalently linked (Fig. 1). Mannitol becomes phosphorylated during transport and is released as mannitol-1-phosphate in the cytoplasm (7). The phosphate group originates from phosphoenolpyruvate (PEP) and is transferred to the various EII sugar translocators in the cytoplasmic membrane via two general PTS proteins, EI and HPr (Fig. 1). The phosphoryl transfer reactions from PEP, via EI, HPr, and the A domain to the B domain of Enzyme II are well understood because three-dimensional structures are available for these proteins combined with detailed pre-steady state kinetic data in the case of the glucose transporter, EII\textsuperscript{bc}, from \textit{E. coli} (14, 15). This type of information is not available for the last phosphorylation step from the B domain to the sugar, bound at the C domain. Moreover, no information is available regarding which residues bind the sugar and which residues facilitate the transport of the sugar.

For a better understanding of how Enzyme II proteins work, detailed structural information about the membrane-embedded C domain is needed. Currently, structural information is limited to a 5-Å two-dimensional projection map of dimeric IIC\textsuperscript{mtl} (16). Enzyme II proteins are known to be functional only as dimers. EII\textsuperscript{mtl} forms very strong dimers when in the bilayer or when solubilized by detergent (7), and careful analysis established that dimeric wt EII\textsuperscript{mtl} shows one high affinity binding site ($K_D = \sim 100 \text{ nM}$) (17). The presence of a second site with a $K_D < 0.1 \text{ mM}$ could be excluded. Because the 5-Å two-dimensional projection map shows a 2-fold symmetry, the location of the single binding site is expected symmetrically positioned in the dimer at the 2-fold symmetry axis.

Here the distances between 19 residue positions in IIC\textsuperscript{mtl} and the mannitol-binding site are presented, as determined by fluorescence resonance energy transfer (FRET) with 5-fluorotryptophan (5-FTrp) as donor and azi-mannitol as acceptor (supplemental Fig. S1) (18). This is an attractive donor-acceptor pair because it shows a short Förster distance ($R_0$) of 9.6 Å, ideal for measuring distances in the 7–16-Å range. 5-FTrp was chosen.

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\footnotesize{2}The abbreviations used are: PTS, phosphoenolpyruvate-dependent group translocation system; 5-FTrp, 5-fluorotryptophan; TMH, transmembrane helix; mannitol, D-mannitol; PEP, phosphoenolpyruvate; FRET, Förster resonance energy transfer; wt, wild type; MS/MS, tandem mass spectrometry.
rather than Trp because of its homogeneous fluorescent decay when incorporated into a protein (19, 20). Because EIImtl is dimeric and harbors only one high affinity mannitol-binding site/dimer (17), azi-mannitol bound at the mannitol-binding site can in principle show FRET with one or both 5-FTrps in the dimer. Comparison of the time-resolved fluorescence decays of 5-FTrp labeled EIImtl in the presence of mannitol versus azi-mannitol informs whether azi-mannitol shows FRET with one or both 5-FTrp residues. The extent of lifetime quenching by azi-mannitol allows calculating both azi-mannitol to 5-FTrp distances in the dimer. Using this approach, the position of the mannitol-binding site with respect to transmembrane helix I could be established as well as its close proximity to residues in IICmtl, suggested before to be involved in mannitol translocation. Our data clearly indicate that the binding site is asymmetrically positioned in the dimer, and this result is discussed together with the available two-dimensional projection maps of IICmtl.

The group translocating transporters are the only class of transporters that chemically modify their substrate concomitant with their transport, and in this work we addressed for the first time where this chemical step takes place. For this, we compared the position of mannitol in unphosphorylated EIImtl with that in phosphorylated EIImtl. FRET experiments with single-Trp mutants, unphosphorylated or stably phosphorylated at the B domain, showed that phosphorylation does not induce changes in the FRET efficiency, demonstrating that mannitol binding and its phosphorylation take place at the same location in IICmtl.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—C$_{10}$E$_5$ detergent was from Kwant High Vacuum Oil Recycling and Synthesis (Bedum, The Netherlands). Fluorescent impurities were removed as described (21).

**Construction of EIImtl Mutants**—All single-Trp mutants except W38$^3$ were based on Trp-less EIImtl with a N-terminal His$_6$ tail and were constructed as described before (22). Mutant W38 was based on Trp-less EIImtl, a gene that was extended by 45 bp at the C terminus (CTGGTGCCGGCGCCTGGGGCAGCAGCCATCACCATC), encoding a tombine cleavage site and a His$_6$ tail. The C384S mutation was introduced in the single-Trp mutants by exchanging the 1100-bp SnaBI-XbaI fragment of the pMaHismltAP, plasmid, encoding the single-Trp mutants (22) with the pMa384S plasmid encoding EIImtl (C384S) (23).

**EIImtl Production, Purification, and Characterization**—Bio-synthetic incorporation of 5-FTrp was performed as described (24) using the M5219 *E. coli* Trp auxotrophic strain (25). Purification of the proteins to homogeneity, according to SDS-PAGE gel electrophoresis, followed a detailed published procedure (22, 26). The nonvectorial phosphorylation activity, catalyzed by EIImtl, was measured as described (27). The mannitol dissociation constants ($K_D$) of 13 single-Trp mutants have been reported previously and showed values similar to wild type ($K_D = ~100$ nM) except W97 ($K_D = 2$ μM) and W198 ($K_D = 375$ nM) (28, 29). For six other mutants the following $K_D$ values were measured as described (30): W38, 67 nM; W180, 60 nM; W251, 120 nM; W260, 190 nM; W282, 77 nM; and W327, 51 nM. EIImtl shows a $K_D$ of 50–100 μM for azi-mannitol (18), and to saturate the EIImtl binding site with this acceptor, a concentration of 1 mM was used (18).

To ensure complete phosphorylation of Ser$^{384}$ in the C384S mutants, the inside-out vesicles were incubated with 5 mM MgCl$_2$, 1 μM HPr, 50 nM EI, and 0.25% C$_{10}$E$_5$ at 30 °C for 20 min, before starting the purification procedure. The solubilization procedure of mutant W260 C384S was slightly different than the one used for other mutants. For this mutant the solubilizing buffer was as follows: 25 mM Tris-HCl, pH 7.6, 10 mM imidazole, 400 mM NaCl, and 1% decylmaltoside. The vesicles were slowly added to 4 volumes of solubilizing buffer and stirred on ice for 20 min.

**Fluorescence Spectroscopy**—All of the fluorescence experiments were performed in buffer I containing 20 mM Tris-HCl, pH 8.4, 250 mM NaCl, 1 mM reduced glutathione, and 0.25% (v/v) C$_{10}$E$_5$, a buffer in which EIImtl is present as dimers (7). EIImtl concentrations of 1–5 μM were used. The emission spectra were recorded on a Fluorolog 3-22 fluorospectrometer (Jobin Yvon) at 23 °C. Excitation was at 295 nm, and excitation and emission bandwidths were 1.25 and 5 nm, respectively. Fluorescence intensities were calculated via integration of the emission peak. In each condition the spectra were recorded two or three times, and the variation between the integral values was found to be less than 5%. All of the spectra were corrected for fluorescence from the buffer and for instrument response.

Fluorescence lifetime (time-correlated single photon counting) measurements were performed at 20 °C as described in detail recently (31). The data were analyzed with a model of discrete exponentials $I(t) = \sum_i \alpha_i \exp(-t/\tau_i)$, where $\alpha_i$ indicates the weight of the component with lifetime $\tau_i$, using the time-resolved fluorescence analysis data processing package, version 1.2, of Scientific Software Technology Center (Belarusian State University, Belarus). Support plane analysis of the fit parameters was performed and gave relatively small errors, a
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result of the strong decay signals collected and the homogeneous decay of 5-FTrp.

The Förster distance \( R_0 \) for the 5-FTrp containing mutants and azi-mannitol was calculated using the corrected emission spectra of the mutants and the absorption spectrum of azi-mannitol. The overlap integral \( (I) \) was calculated using Origin (Microcal) software. The values for the refractive index \( (n) \) is 1.6 (32) and orientation factor \( (k^2) \) is 0.666 were used. Because of the low solubility of ElI_{mtl}, the quantum yield \( (Q) \) was not measured directly but was estimated using the fluorescence lifetime \( (\tau) \) of the mutants and the natural lifetime of N-acetyl-5-fluorotryptophan-amine (21 ns) (19). Despite some variability in \( I \) and \( Q \) for all of the mutants harboring a 5-FTrp residue in close proximity of azi-mannitol \( (r \approx 7–8 \text{ Å}) \), multiple lifetimes were observed and also part of \( \alpha \) “disappeared,” because a fraction of \( \tau_{DA} \) was below the detection limit \( (\tau_{DA} < 0.2 \text{ ns, corresponding with } r \leq 6 \text{ Å}) \). In these cases \( \alpha, \tau_D \) and the amplitude averaged lifetime \( <\tau_{DA}> = \left(\Sigma\alpha_i\tau_i\right) \) instead of \( \tau_D \) and \( \tau_{DA} \), respectively, were used to calculate \( E \) and \( r \).

**RESULTS**

**Location of the Mannitol-binding Site with Respect to Transmembrane Helix I**—Topology models of IIC_{mtl} show six to eight transmembrane helices (TMHs) (34–36), and all available topology models agree on the presence of the residues forming TMH1, TMH2, and the last TMH of IIC_{mtl} (Fig. 2) (6). TMH1 contains residues 20–42, and it is the most conserved TMH in IIC_{mtl} (6). Three single-Trp ElI_{mtl} mutants were biosynthetically labeled with 5-FTrp at positions 30, 38, and 42 (mutants W30, W38, and W42, respectively). Comparison of the fluorescence decay of each dimeric mutant, in the presence of mannitol (donor only fluorescence) or with azi-mannitol, informs about the FRET efficiency \( (E) \) between the two 5-FTrp probes and azi-mannitol. High FRET efficiencies were observed for W30 and W38. In the presence of mannitol, both 5-FTrp probes in W30 show a fluorescence lifetime \( (\tau) \) of 4.5 ns as a homogeneous decay was observed (supplemental Fig. S2 and Table 1). The amplitude averaged fluorescence lifetime \( <\tau> \) \( = \left(\Sigma\alpha_i\tau_i\right) \) of W30 with mannitol decreases 39% when it binds azi-mannitol. This decrease is in good agreement with the observed decrease in steady state emission intensity \( (\sim 37\%\); Table 1). In the presence of azi-mannitol, the \( \tau \) of one 5-FTrp is quenched by FRET. This is visible in the decay curve as a sharp drop in intensity (supplemental Fig. S2). Using Equations 1 and 2, it can be calculated (see “Experimental Procedures”) that one 5-FTrp is 7–8 Å from the mannitol-binding site \( (E = \sim 0.8) \), whereas the other 5-FTrp is \( \geq 16 \text{ Å} \) apart from the mannitol-binding site \( (E \leq 0.05) \).

For mutant W38 in the presence of mannitol, 50% of \( \alpha \) is for \( \tau = 5.3 \text{ ns} \), and replacement of mannitol by azi-mannitol does...
not have an effect on this $\alpha$, whereas $\tau$ slightly decreases from 5.3 to 4.9 ns, indicating that this 5-FTrp is 15 A away from the mannitol-binding site. The $<\tau>$ of the other 5-FTrp reduces 80% when azi-mannitol is present. Thus this residue is close to the mannitol-binding site (7–8 Å).

The two 5-FTrp probes in mutant W42, incubated with mannitol, show lifetimes of 3.9 and 5.3 ns, respectively. When mannitol is replaced by azi-mannitol, the $\tau$ of one 5-FTrp reduces 13%, whereas the $\tau$ of the other 5-FTrp remains essentially the same (supplemental Fig. S2 and Table 1). According to Equations 1 and 2, one 5-FTrp is 13 Å, whereas the other is $\geq$16 Å apart from the mannitol-binding site.

A model of TMHII was built including the I38W mutation. Trp rotamers were chosen according to the most favorable orientation given by the program COOT that uses the rotamer library of Lovell et al. (37). Three spheres were drawn, two with radii of 7.5 Å and one with a radius of 13 Å, centered at the C$\beta$ atoms of the indole rings at positions 30, 38, and 42, respectively. The surfaces of the three spheres closely approach each other at two areas $\sim$2 and $\sim$10 Å apart from the central helix axis (Fig. 3). The nearest position is occupied by amino acids of the helix, making this position unsuitable as putative mannitol-binding site. This leaves the other position as the mannitol-binding site. This site is located in the middle of TMHII and faces residue 34.

Location of Residues 70–134 with Respect to the Mannitol-binding Site—In the first published IIC$^{mtl}$ topology model of Sugiyama et al. (34) residues 70–134 form a cytoplasmic loop connecting TMHII and TMHIII. Recent Trp fluorescence and phosphorescence spectroscopy data (29, 31) and cysteine accessibility studies (35) show that this part of IIC$^{mtl}$ is highly structured. Indeed, in recent topology models these residues

| $\tau_1$ | $\tau_2$ | $\tau_3$ | $\alpha_1$ | $\alpha_2$ | $\alpha_3$ | $\chi^2$ | Decrease in $<\tau>$ mtl → azi-mtl | Decrease in steady-state emission mtl → azi-mtl |
|---------|---------|---------|-----------|-----------|-----------|--------|-----------------------------|----------------------------------|
| W30 + mtl | 1.8 | 4.5 | ns | 0.05 | 0.95 | 0.98 | 39 | 37 |
| + azi-mtl | 0.3 | 1.8 | 4.5 | 0.17 | 0.18 | 0.48 | 1.04 | 1.06 |
| W30C384S + mtl | 4.3 | 7.7 | 0.96 | 0.04 | 1.06 | 38 | 36 |
| + azi-mtl | 0.3 | 1.4 | 4.3 | 0.14 | 0.16 | 0.55 | 0.95 | 1.10 |
| W38 + mtl | 0.6 | 3.7 | 5.3 | 0.07 | 0.43 | 0.50 | 1.00 | 36 |
| + azi-mtl | 0.3 | 2.2 | 4.9 | 0.15 | 0.14 | 0.49 | 1.00 | 36 |
| W42 + mtl | 1.0 | 3.9 | 5.3 | 0.04 | 0.47 | 0.48 | 1.12 | 11 |
| + azi-mtl | 0.9 | 3.4 | 5.1 | 0.06 | 0.44 | 0.48 | 1.04 | 14 |
| W42C384S + mtl | 0.7 | 4.0 | 5.4 | 0.03 | 0.46 | 0.51 | 1.11 | 14 |
| + azi-mtl | 0.9 | 3.2 | 5.1 | 0.06 | 0.42 | 0.47 | 1.17 | 17 |
| W97 + mtl | 4.6 | 8.6 | 0.98 | 0.02 | 1.11 | 33 | 37 |
| + azi-mtl | 1.5 | 4.4 | 0.33 | 0.61 | 1.04 | 33 | 37 |
| W133 + mtl | 1.0 | 4.5 | 7.6 | 0.09 | 0.90 | 0.01 | 1.09 | 39 |
| + azi-mtl | 1.5 | 4.6 | 7.6 | 0.26 | 0.51 | 1.03 | 39 | 33 |
| W188 + mtl | 4.0 | 5.8 | 0.5 | 0.5 | 1.05 | 24 | 20 |
| + azi-mtl | 1.4 | 2.5 | 5.3 | 0.19 | 0.29 | 0.52 | 1.06 | 16 |
| W198 + mtl | 4.2 | 5.7 | 0.5 | 0.5 | 1.07 | 16 | 16 |
| + azi-mtl | 4.0 | 4.9 | 0.5 | 0.5 | 1.07 | 16 | 16 |
| W251 + mtl | 2.1 | 4.4 | 0.03 | 0.97 | 1.00 | 1.00 | 1.00 |
| + azi-mtl | 0.6 | 4.0 | 0.04 | 0.97 | 1.00 | 1.00 | 1.00 |
| W251C384S + mtl | 2.6 | 4.5 | 0.08 | 0.92 | 1.08 | 1.08 | 1.08 |
| + azi-mtl | 2.2 | 4.3 | 0.07 | 0.92 | 1.06 | 7 | 6 |
| W260 + mtl | 0.3 | 2.1 | 4.9 | 0.20 | 0.22 | 0.58 | 1.10 | 8 |
| + azi-mtl | 4.4 | 0.69 | 0.99 | 11 | 5 |
| W260C384S + mtl | 0.5 | 2.2 | 4.9 | 0.06 | 0.23 | 0.71 | 1.01 | 8 |
| + azi-mtl | 1.1 | 3.3 | 5.6 | 0.04 | 0.46 | 0.37 | 1.03 | 3 |

* The addition of azi-mannitol resulted in an increase in amplitude due to a red shift of the emission spectrum (see text).
* Not calculated because of a spectral shift induced by azi-mannitol binding (see text).
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are predicted to form a TMH and a periplasmic loop (36) or protrude into the membrane from the cytoplasmic side (Fig. 2) (35). Mannitol binding or phosphorylation of IIA<sup>mtl</sup> and IIB<sup>mtl</sup> domains induce conformational changes in this part of IIC<sup>mtl</sup> (29, 35, 38). These results suggest that residues in this part of IIC<sup>mtl</sup> are directly involved in the mannitol translocation process. If so, short distances between certain residues in this part and the mannitol-binding site are expected.

Experiments with single-Trp mutants W109 and W117 showed that no FRET takes place between these Trps and azi-mannitol (18). Thus Trp 109 and 117 in both subunits of dimeric EII<sup>mtl</sup> are positioned ≥16 Å apart from the mannitol-binding site. For W114 and W126, labeled with 5-FTrp, also no FRET was observed, indicating that these residues in both subunits of the dimer are also ≥16 Å away from the mannitol-binding site. The FRET data for W97 show that one 5-FTrp is close to the mannitol-binding site (7–8 Å, E = 0.8), whereas the 5-FTrp in the other subunit is ≥16 Å away. Similar distances were measured for mutant W133, because one 5-FTrp residue becomes efficiently quenched by azi-mannitol (E = 0.8, r = 7–8 Å), whereas the r and α of the other 5-FTrp remain unchanged (supplemental Fig. S3 and Table 1). In conclusion, the short distances obtained with mutants W97 and W133 underpin the view that residues in the range 70–134 are involved in the mannitol translocation process.

Location of Two Conserved Motifs in EII<sup>mtl</sup> Critical for Functioning—The GLXE motif is found in all EII sugar transporters, and the glutamic acid is fully conserved (36). In EII<sup>mtl</sup> this motif constitutes residues Gly<sup>254</sup>-Ile<sup>255</sup>-His<sup>256</sup>-Glu<sup>257</sup>. At a constant distance upstream of the GLXE motif, two conserved hydrophilic residues are found in the sequence of EIImtl (6). In EIImtl these residues are Asn<sup>194</sup> and His<sup>195</sup>. Mutation studies with EIImtl showed that both motifs are critical for mannitol binding and mannitol transport, and all reported mutations introduced in these motifs abolished the high mannitol binding affinity (39–42).

To study the location of these motifs with respect to the mannitol-binding site, Phe<sup>198</sup>, Phe<sup>251</sup>, and Phe<sup>260</sup>, flanking these motifs, were mutated to a 5-FTrp in Trp-less EIImtl (mutants W198, W251, and W260, respectively). The affinity for mannitol binding in W251 was found to be comparable with wt (K<sub>d</sub> = 120 nM), whereas for W198 and W260 the mannitol affinity had slightly decreased (K<sub>d</sub> = 375 and 190 nM, respectively).

W260, labeled with 5-FTrp, shows complex fluorescence decay kinetics in the presence of mannitol. Binding of azi-mannitol induced an 11% decrease of <τ>, but because of the complex decay, this decrease could not be translated into distance information between the two 5-FTrp probes and the mannitol-binding site. Also for mutant W251, small but reproducible effects of azi-mannitol on the fluorescence in time-resolved (∼3%) and steady state (∼5%) modes were observed.

In the presence of mannitol, the decay of W198 showed two lifetimes with equal amplitudes (4.2 and 5.7 ns) (Table 1). When azi-mannitol was present, one τ minimally changed (∼5%), whereas the other τ dropped by 14%. Remarkably, replacing mannitol by azi-mannitol not only resulted in a 16% drop in emission intensity but also induced a red shift of the emission spectrum, suggesting that the change in substrate induced a structural alternation near 5-FTrp198. The red shift gave an increase in α because the emission is shifted to the bandwidth of the interference filter used (supplemental Fig. S3 and Table 1). Translated into distances, one 198 position is 13 Å (E = 0.14) away from the binding site, whereas the other is 16 Å (E = 0.05) away.

Taken together, the FRET results for positions 198, 251, and 260 indicate they are not close to where mannitol is bound. Because we had to study flanking positions, we cannot exclude that the residues constituting the motifs are closer to the mannitol-binding site.

Positions of Residues 66, 147, 167, 180, 188, 282, and 327 with Respect to the Mannitol-binding Site—To investigate the proximity of other parts of IIC<sup>mtl</sup> near the mannitol-binding site, 5-FTrp was introduced at seven more positions (Fig. 2). In each case a conservative Phe → Trp mutation (Tyr → Trp for position 66) was introduced in Trp-less protein at positions 66, 147, 167, 180, 188, 282, and 327, yielding mutants W66, W147, W167, W180, W188, W282, and W327, respectively. None of these positions have been related before to the activity of EIImtl. Only mutant W188 showed quenching by azi-mannitol (supplemental Fig. S3 and Table 1). One 5-FTrp residue is in a position 9 Å from the binding site (E = 0.54), whereas the other 5-FTrp residue is at a distance of 14 Å (E = 0.10).

Position of the Mannitol-binding Site in Phosphorylated EII<sup>mtl</sup>—Unphosphorylated EIImtl facilitates transport of mannitol over the membrane. This transport activity increases 2–3 orders of magnitude if the enzyme is phosphorylated (43, 44). Calorimetry studies revealed that strong B/C domain-domain interactions are induced upon phosphorylation of Cys<sup>384</sup> in the B domain (45). It is not clear whether enzyme phosphorylation induces movement of mannitol to the cytoplasmic side of IIC<sup>mtl</sup>, where Cys<sup>384</sup> phosphorylates it, or that enzyme phosphorylation triggers movement of the phosphorylated B domain toward the mannitol-binding site in the C domain. To address this aspect of the mannitol translocation mechanism, single-Trp mutants carrying the C384S mutation were constructed. Previously it was shown that Ser<sup>286</sup> becomes irreversibly phosphorylated when PEP, EI, and HPr are present (46, 47). Because the phosphoryl group cannot be transferred to mannitol, these mutants show no mannitol phosphorylation activity, but the mannitol affinity is not affected by this mutation (23). Thus C384S mutants are ideal models to study substrate-bound EII<sub>mtl</sub> in its activated phosphorylated state (23, 47).

The C384S mutation was introduced in mutants W30 and W42, and both mutants were biosynthetically labeled with 5-FTrp. Mass spectrometry was used to confirm that Ser<sup>286</sup> was phosphorylated. After its characterization by fluorescence, as described below, the purified C384S mutant was treated with trypsin, and all of the peptides were analyzed by nano-flow reversed phase high performance liquid chromatography MS/MS on a linear quadrupole ion trap-Fourier transform hybrid mass spectrometer (LTQ Orbitrap XL). The MS/MS data were matched to the sequence of the EII<sub>mtl</sub> mutant with the program Mascot (48), including oxidation of methio-
nine, deamidation of glutamine and asparagine, and phosphorylation of serine and threonine as a variable modification. The 380–399 peptide was detected exclusively in the phosphorylated form, with a deviation of <0.003 Da from the theoretical mass. MS/MS experiments identified Ser384 as the phosphorylation site (supplemental Fig. S5). Based on these results, we concluded that >95% of Ser384 had become phosphorylated.

The FRET experiments show that the effect of azi-mannitol on the fluorescence is the same as observed for these two mutants not carrying the C384S mutation (supplemental Figs. S2 and S4 and Table 1). For example, for mutant W42, with its 5-FTrp at the periplasmic end of TMH1 (Fig. 2), a weak FRET efficiency of $E = 0.13$ was observed with both samples. If enzyme phosphorylation resulted in movement of the mannitol-binding site toward the cytoplasmic side, FRET was no longer expected.

Two other mutants, W251 C384S and W260 C384S, confirmed that phosphorylation of EIImtl does not change the position of mannitol in IICmtl. For both mutants, the same degree of quenching by azi-mannitol was observed in the absence and presence of the phosphorylated 384 position (supplemental Fig. S4 and Table 1). Thus phosphorylation of the B domain does not induce movement of mannitol toward the cytoplasmic site.

**DISCUSSION**

With the elucidation of the high resolution three-dimensional structure of several transport proteins in recent years, a new phase in membrane transport research has been entered. A common feature of these structures is that the solute has to diffuse to a centrally located binding site. For the few transport proteins that could be crystallized at different states of the transport cycle, models showing the step-by-step translocation of the solute have been presented. For example, for members belonging to the betaine/choline/carnitine transporters, structural evidence has been provided that the solute-binding site becomes occluded before the solute is expelled at the other side of the bilayer (49, 50). For transporters, for which no three-dimensional structures are available, relatively little information is available about the location of the substrate-binding site and even the stoichiometry of the number of binding sites per functional oligomer.

An attractive approach to locate the binding site is to measure distances between the substrate in the binding site and residues in the (oligomeric) transporter. A FRET approach can in principle give this information, provided that the introduction of the donor-acceptor pair does not perturb the transporter-substrate interaction and that no long linkers are needed to introduce the chromophores, to enhance the reliability of the measurements. To accurately measure distances close to the lower FRET distance limit of $\sim 5$ Å (51), a donor-acceptor pair with a $R_0$ of $\leq 10$ Å is needed, but such pairs are rare.

To establish the location of the mannitol-binding site in EIImtl, we introduced the Trp-azi-mannitol donor-acceptor pair ($R_0$ of 9.8 Å) (18). The small size of the diazirine moiety, a mere two nitrogen atoms, makes it ideal for incorporation in biological ligands, without introducing large changes in biological activity. Indeed, azi-mannitol competes for the same binding site as mannitol (18), and azi-mannitol is transported by EIImtl (52).

Because of the complex fluorescence decay of Trp, previous studies on the localization of the mannitol-binding site in EIImtl with single-Trp mutants could not establish whether one or both Trp residues showed FRET with azi-mannitol (18). However, the Trp analog 5-FTrp shows a homogeneous decay at most protein positions analyzed so far (19, 31, 53, 54), making it suitable to study distances in an oligomeric protein. With azi-mannitol as acceptor, a $R_0$ of 9.6 Å is obtained, and here for the first time a systematic FRET study is presented, taking advantage of the homogeneously decaying 5-FTrp as donor. Of the 19 donor IICmtl positions investigated, nine showed FRET, demonstrating that at least one 5-FTrp in these mutants is <16 Å away from the mannitol-binding site.

Because the structure of the analyzed donor-acceptor complexes is not known, the obtained distances should be handled with some caution because no information is available about the relative orientation of the chromophores, expressed in $k^2$, thus introducing an uncertainty in the calculated distance. Due to the rather low intrinsic anisotropy value of 0.23, when 5-FTrp is excited at 305 nm (20), this uncertainty in the distance related with $k^2$ could be significantly reduced, likely to <10% (33, 55). Moreover, all of the mutants except two (W30 and W97) show subnanosecond anisotropy decay components (31), and the acceptor is expected to be mobile on the fluorescence time scale, given its small size of 28 Daltons.

For most mutants the emission properties of the individual 5-FTrp residues in the dimer could be distinguished, which is a prerequisite to calculate both donor-acceptor distances. For some mutants, like W260 and W260 C384S, the fluorescent decay was too complex to calculate both distances, but inspection of the changes in $r$ and steady state emission allowed us to make a qualitative measure of the FRET efficiency and thus obtain information on these distances.

The FRET results with mutants W30, W38, and W42 revealed the position of the binding site in the middle of TMH1 (residues 20–42), facing residue 34 (Fig. 3). Because all available IICmtl topology models predict this part of the transporter as an $\alpha$-helix of ≈23 residues long, it is likely a TMH. Thus, as for the primary and secondary transporters, of which three-dimensional structures have been solved, the substrate-binding site is situated approximately in the middle of the membrane in this transporter. Residues 97 and 133 are in close proximity (7–8 Å) with this binding site. Characterization of mutants carrying a mutation near position 30, 38, 97, or 133 can further refine our picture of the mannitol-binding site. Introduction of a Trp at nine other TMH1 positions (single-Trp mutants W25, W27, W28, W32, W33, W34, W35, W37, and W40) yielded mutants showing affinity for mannitol and mannitol phosphorylation activities as found for wt and Trp-less EIImtl. Thus TMH1 residues are not directly involved in

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4. M. Opačić, E. P. P. Vos, B. H. Hesp, and J. Broos, unpublished results.
5. G. Veldhuis and E. P. P. Vos, unpublished results.
Mannitol-binding Site in EI\textsuperscript{mnt}

mannitol binding. Unfortunately, these mutants were too unstable to be purified.

EI\textsuperscript{mnt} mutants carrying a single Cys in the IIC\textsuperscript{mnt} domain at positions 85, 90, 91, 92, 93, 100, 124, and 136 have been reported (35, 56). The introduction of the T85C, V90C, G91C, and S134C mutations reduced the affinity for mannitol, whereas in the case of the I100C mutation, the mannitol affinity was completely abolished. These results are in line with this and previous studies that this part of IIC\textsuperscript{mnt} is involved in mannitol translocation. We note that of the dozens of IIC\textsuperscript{mnt} mutants reported, only a few show a lower mannitol affinity. Apart from the Cys mutants just discussed and mutant W97, mutations introduced in the two conserved motifs were found to strongly reduce the binding affinity (7) as well as mutant E218V (57).

The obtained distances in this work shed new light on how mannitol is translocated by dimeric EI\textsuperscript{mnt}. Significant is the finding that phosphorylation of the B domain does not induce movement of mannitol toward the cytoplasmic side of IIC\textsuperscript{mnt}, in this way facilitating phosphoryl transfer from the B domain. The presented data show that the B domain has to move to this binding site in IIC\textsuperscript{mnt}, after accepting the phosphoryl group from the A domain. Movement of the B domain to this site suggests that extensive interactions between the C and B domains are taking place, a view supported by calorimetry measurements, which indicated that up to 50–60 residues at the B/C domain interface are involved in this interaction (45).

Another valuable result is the observation that the mannitol-binding site is asymmetrically positioned in dimeric IIC\textsuperscript{mnt}. For mutants W30, W38, W97, and W133, one 5-FTrp residue was found within 7–8 Å of the mannitol-binding site, whereas for the 5-FTrp in the other subunit a distance ≥15 Å was measured. These differences in distances are too large to be caused by the asymmetric positioning of the diazirine group at the C2 position rather than the central C3 position of the azi-mannitol molecule (supplemental Fig. S1).

After the report that only one binding site could be detected in dimeric EI\textsuperscript{mnt} (17), this site was expected at the putative 2-fold symmetry axis in a 5-Å projection map of dimeric IIC\textsuperscript{mnt} obtained after merging EM data from different two-dimensional crystals and imposing 2-fold symmetry. However, in a 7-Å projection map of IIC\textsuperscript{mnt}, obtained from a single crystal and grown in the presence of mannitol, small differences in solvent accessibility of the two 5-FTrp probes per dimer were interpreted as an indication of structural asymmetry. A homogeneous fluorescence decay together with differences in solvent accessibility of the two 5-FTrp probes per dimer have also been reported for mutants W97 and W133 and were also interpreted as an indication of structural asymmetry in dimeric IIC\textsuperscript{mnt} (31). When homodimeric IIC\textsuperscript{mnt} shows nearly P2\textsubscript{2} symmetry, and the residues, forming the high affinity binding site, do not cluster together at the P2\textsubscript{2} axis, these residues also cluster together at the symmetry-related position. We suggest that this location is a second "resting" mannitol-binding site.

Kinetic studies with EI\textsuperscript{mnt} revealed the presence of two populations of binding sites/EI\textsuperscript{mnt} dimer with distinct kinetic properties, and it was proposed that they are oriented in opposite directions in the dimer (58). Moreover, it has been demonstrated that the periplasmic side of EI\textsuperscript{mnt} shows a much higher affinity for mannitol than its cytoplasmic side (43). These results from kinetic studies, combined with the stoichiometry data of one high affinity binding site/dimer (17) and the structural information presented in this work, lead to the following transport model for EI\textsuperscript{mnt}, the loaded high affinity site, after releasing mannitol-1-phosphate in the periplasm, is converted to a low affinity resting binding site, a process coupled with a gain in mannitol affinity of the resting site in the other protomer, because it transfers from a low affinity cytoplasmic oriented site to a high affinity site ready to bind mannitol from the periplasm.

In conclusion, this work demonstrates the potential in structural and mechanistic protein research of a donor-acceptor pair with a very short R\textsubscript{0} of which the donor shows homogeneous fluorescence decay kinetics. Also attractive is the straightforward biosynthetic incorporation of the donor at either buried or surface-exposed positions. Despite the fact that a rather complex protein system was investigated, an oligomeric transport protein, important new information was obtained, including the localization of the binding site at different stages of the transport cycle and details of the oligomeric organization of this transporter. The present approach offers an attractive tool to study other proteins, in particular those proteins for which a three-dimensional structure cannot be easily obtained.

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