A Fluorescence Study of Single Tryptophan-Containing Mutants of Enzyme II\textsuperscript{mtl} of the Escherichia coli Phosphoenolpyruvate-Dependent Mannitol Transport System\textsuperscript{†}

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ABSTRACT: The fluorescence properties of six different single Trp mutants of the mannitol-specific transporter of Escherichia coli, enzyme II\textsuperscript{mtl} (EI\textsuperscript{mtl}),\textsuperscript{1} is a member of the P-enolpyruvate-dependent phosphotransferase (PTS) system. The enzyme is responsible for the transport and phosphorylation of mannitol from the periplasm to the cytoplasm in Escherichia coli. It consists of a hydrophobic membrane-embedded C domain possessing the mannitol binding and translocation site and two cytoplasmic domains, A and B. The enzyme and the separate domains have been cloned, overexpressed, and purified to homogeneity (Robillard et al., 1993; Van Weeghel et al., 1991; Boer et al., 1994). The 3D structures of the cytoplasmic A and B domains are currently being solved by X-ray crystallography and NMR spectroscopy (Kroon et al., 1993; AB et al., 1994; van Montfort et al., 1994). Unfortunately, these techniques cannot be readily used to solve the structure of the 35 kDa membrane-embedded C domain. However, the four tryptophan residues present in the C domain can, in principle, be used as spectroscopic probes to provide valuable information about the structure and dynamics of this domain. The information is limited, in the case of multiple tryptophans, due to overlapping emission spectra, energy transfer between the tryptophans, and multiple lifetimes for each tryptophan. For this reason, we constructed a series of single tryptophan mutants, replacing the natural tryptophans with phenylalanines. Initial spectral characterizations were plagued by artifacts arising from fluorescent impurities with emission maxima in the range of 320–400 nm, the same range where the tryptophan emission signal was expected. These observations prompted us to investigate the source of the impurities, which was mainly from commercial detergents. A tryptophan-minus mutant of EI\textsuperscript{mtl} was created to develop methods to remove or avoid the impurities. The exercise was judged successful only when an EI\textsuperscript{mtl}(Trp\textsuperscript{−}) fluorescence spectrum could be routinely obtained showing characteristic tyrosine fluorescence and no significant fluorescence in the tryptophan at position 30.

The mannitol-specific transport protein of Escherichia coli, enzyme II\textsuperscript{mtl} (EI\textsuperscript{mtl}),\textsuperscript{1} is a member of the P-enolpyruvate-dependent phosphotransferase (PTS) system. The enzyme is responsible for the transport and phosphorylation of mannitol from the periplasm to the cytoplasm in Escherichia coli. It consists of a hydrophobic membrane-embedded C domain possessing the mannitol binding and translocation site and two cytoplasmic domains, A and B. The enzyme and the separate domains have been cloned, overexpressed, and purified to homogeneity (Robillard et al., 1993; Van Weeghel et al., 1991; Boer et al., 1994). The 3D structures of the cytoplasmic A and B domains are currently being solved by X-ray crystallography and NMR spectroscopy (Kroon et al., 1993; AB et al., 1994; van Montfort et al., 1994). Unfortunately, these techniques cannot be readily used to solve the structure of the 35 kDa membrane-embedded C domain. However, the four tryptophan residues present in the C domain can, in principle, be used as spectroscopic probes to provide valuable information about the structure and dynamics of this domain. The information is limited, in the case of multiple tryptophans, due to overlapping emission spectra, energy transfer between the tryptophans, and multiple lifetimes for each tryptophan. For this reason, we constructed a series of single tryptophan mutants, replacing the natural tryptophans with phenylalanines. Initial spectral characterizations were plagued by artifacts arising from fluorescent impurities with emission maxima in the range of 320–400 nm, the same range where the tryptophan emission signal was expected. These observations prompted us to investigate the source of the impurities, which was mainly from commercial detergents. A tryptophan-minus mutant of EI\textsuperscript{mtl} was created to develop methods to remove or avoid the impurities. The exercise was judged successful only when an EI\textsuperscript{mtl}(Trp\textsuperscript{−}) fluorescence spectrum could be routinely obtained showing characteristic tyrosine fluorescence and no significant fluorescence in the tryptophan region.
**EXPERIMENTAL PROCEDURES**

**Materials.** Sodium deoxycholate (DOC) was obtained from Sigma. Decylpentae(ethylene glycol) (C₁₀E₅) and decylpoly(ethylene glycol) 300 (decyl-PEG) were a gift from Kwant High Vacuum Oil Recycling and Synthesis, Bedum, The Netherlands. Q-Sepharose Fast Flow and S-Sepharose Fast Flow were from Pharmacia (Sweden); hexyl-agarose was from Sigma. d-1-[¹⁴C]Mannitol (59 mCi/mmol; 1 mCi = 37 MBq) was purchased from the Radiochemical Center Amersham; d-1-[⁷H(N)]mannitol (976.8 GBq/mmol) was obtained from DuPont NEN Research Products. Hexane was distilled from P₂O₅. Uvasol isooctane from Merck was further purified by passage through a silica column. All other reagents were analytical grade.

**Bacterial Strains and Plasmids.** The *E. coli* bacterial strain LGS-322 [F− thi-1, hisG1, argG6, metB1, tonA2, supE44, rpsL104, lacY1, galT6, galR49, gat50, Δ(mt1A), p, mtld, Δ(gutR)MDBA-recA)] (Grisafi et al., 1989), containing a chromosomal deletion in the wild-type mtlA gene, was used for the expression of the mutants of EII\textsuperscript{mtl} as well as for the wild-type EII\textsuperscript{mtl}. The four single tryptophan-containing mutants, where the tryptophan residues were located at their natural positions in the sequence (residue 30, 42, 109, or 117), were constructed by the Kunkel method (Kunkel, 1985) using mutagenic primers to replace the other three tryptophan residues by phenylalanines. The primers were W30F, 5′ GAT GAT ACC AAA CGC GAT AAA 3′; W42F, 5′ GTT CGG AAA CCC TGT TGG 3′; W109F, 5′ CCT AAT GCA AAA GCC GGC CAG 3′; and W117F, 5′ ACC GTC TAC AAA GCG GTC GAA 3′.

Two single tryptophan mutants, EII\textsuperscript{mtl}(Trp−) C320W and EII\textsuperscript{mtl}(Trp−) C384W, were created in the tryptophan-minus construct. The primers used were EII\textsuperscript{mtl}(Trp−) C320W, 5′ CC GCC GTC CCA GGC AAC GAT G 3′; and EII\textsuperscript{mtl}(Trp−) C384W, 5′ AT CGC CGC CCA AAC GAT G 3′.

All constructs were completely sequenced and, except for the intended change and a silent mutation in the codon of glycine 289 (GGT → GCC) in the EII\textsuperscript{mtl}(WFF) mutant, were found to agree with the wild-type sequence (Lee & Saier, 1983).

**Purification of Detergent.** C₁₀E₅ [10% (v/v)] was crystallized from hexane/isooctane [1:1 (v/v)] in a Corex centrifugation tube. Crystals were collected at −5 °C by centrifugation. This procedure was repeated 2–3 times. The final recovery was over 50% by weight of spectrally pure detergent. Fluorescent impurities (>99%), UV-absorbing material, and also aldehydes (Avigad, 1983) were removed by this protocol. DOC was recrystallized twice from acetone/H₂O before use, reducing the amount of emission (excitation 290 nm) to 3% of that found in the starting material.

**Special Precautions Regarding Fluorescent Contaminants.** Water, 3 times distilled in a quartz glass still, was filtered and deionized with a Labconco system (ultrapure water). Glassware, tubing, storage bottles, filters, columns, etc. were checked for fluorescent leakage after contact with buffers containing C₁₀E₅. If necessary, they were pretreated for several days with analytical grade methanol and C₁₀E₅-containing buffer until virtually no fluorescent material was present. Resins were always prewashed with C₁₀E₅-containing buffers until no fluorescence was detected in the elution buffer. Servapar dialysis tubing (Serva) with a cutoff of 12 000–19 000 Da was used for dialysis after heating for 40 min at 100 °C in ultrapure water containing 2 mM Na₂EDTA to remove ultraviolet-adsorbing materials and metal ions (Reynolds et al., 1967). Prior to the dialysis of samples in purified C₁₀E₅, the dialysis tubing was further treated by storage for at least 24 h in C₁₀E₅-containing buffer.

**Protein Purification.** EII\textsuperscript{mtl}(Trp−) and all single tryptophan proteins were purified essentially as described for EII\textsuperscript{mtl}-C384S (Robillard et al., 1993). The only modification was the extra rinsing of all materials to spectroscopically acceptable levels, with detergent-containing buffers and/or analytical grade methanol. Fluorescent backgrounds clearly below the intensity of the Raman peak of water were considered acceptable. Replacement of decyl-PEG by C₁₀E₅ led to altered binding characteristics of EII\textsuperscript{mtl} on hexyl-agarose. Therefore, after washing of the enzyme bound on hexyl-agarose at 4 °C with 1 column volume of extraction buffer [20 mM Tris-HCl, 50 mM NaCl, 3 mM DTT, and 0.5% (v/v) DOC, pH 8.4], an extended wash step at room temperature was carried out with 20 mM Tris-HCl, pH 8.4, 500 mM NaCl, 1 mM DTT, and 0.15% (v/v) C₁₀E₅. The elution was also performed at room temperature using a gradient of 0.15–4% (v/v) C₁₀E₅ in the same wash buffer. The fractions containing the enzyme, which eluted at about 2.5% (v/v) C₁₀E₅, were diluted 5 times with 20 mM Tris-HCl, 1 mM DTT, and 0.25% (v/v) C₁₀E₅, pH 8.4, and directly applied to Q-Sepharose at 4 °C. The Q-Sepharose procedure was identical to the procedure published earlier, except for the replacement of 0.35% (v/v) decyl-PEG by 0.25% (v/v) C₁₀E₅. Enzyme I and HPr were purified as described previously (Dooijewaard et al., 1979; Robillard et al., 1979; van Dijk et al., 1990).

**Mannitol Phosphorylation and Binding.** The PEP-dependent mannitol phosphorylation activity of EII\textsuperscript{mtl} and the different mutants was measured as described (Robillard & Blaauw, 1987). The assay mixture contained 25 mM Tris, pH 7.6, 5 mM MgCl₂, 5 mM DTT, 5 mM PEP, 0.25% (v/v) decyl-PEG, 10 μM HPr, 0.2 μM EI, and 0.5 mM [¹⁴C]-mannitol. Flow dialysis was used to monitor mannitol binding and determine dissociation constants (Lolkema et al., 1990). Specific details are given in the legends and text.

**Concentration Determinations of EII\textsuperscript{mtl} and EII\textsuperscript{mtl} Mutants.** The concentration of EII\textsuperscript{mtl} and the different mutants was determined by the pyruvate burst method (Robillard & Blaauw, 1987), which quantitates the number of phosphorylation sites, or by flow dialysis (Lolkema et al., 1990), which quantitates the number of mannitol binding sites, assuming one high-affinity binding site (Kₐ ~ 100 nM) per EII\textsuperscript{mtl} dimer in accordance with the observations of Pas et al. (1988).

**Fluorescence Measurements.** Samples were dialyzed at 4 °C for at least 3 h in pretreated dialysis tubing with a molecular mass cutoff of 12 000–19 000 daltons, against 20 mM Tris, pH 8.0, 100 mM NaCl, 0.1% (v/v) C₁₀E₅, and 1 mM GSH to eliminate absorption and quenching due to buffer components like oxidized DTT. When studying the phosphorylated enzyme, the samples were first incubated for 5 min at 30 °C in the presence of 5 mM MgCl₂, 5 mM PEP, 1 μM HPr, and 0.02 μM EI. The effect of mannitol binding on the enzyme fluorescence was studied at 5 °C. Spectra were taken of samples containing 0.3 μM (0.02 mg/mL) EII\textsuperscript{mtl} and mutants of EII\textsuperscript{mtl}.

Steady-state fluorescence measurements were performed at 5 °C on a SPF-500C spectrofluorometer (SLM Amino)
operating with a 300 W xenon lamp type LX 300 uv. Emission and excitation spectra were recorded with bandwidths of 4 nm on both monochromators. Spectra were corrected for the buffer base lines (mainly Raman scatter of water) and the instrument response using the appropriate correction curve. More details are given in the figure legends. All samples were stored in liquid nitrogen. Estimates of the percentages of fluorescent species present in different samples were based on the total integrated areas under the emission spectra.

RESULTS

Steady-State Kinetics and Binding Properties of Mutants and Wild-Type EII\textsuperscript{mtl}. EII\textsuperscript{mtl} steady-state kinetics can be characterized by $K_M$ and $V_{\text{max}}$ values extrapolated from the rate dependencies as a function of the concentrations of P-HPr and mannitol, the phosphoryl group donor and acceptor, respectively. Lolkema et al. (1993) reported two kinetic regimes for mannitol phosphorylation at saturated HPr concentrations, leading to a high-affinity and a low-affinity $K_M$. The high-affinity (HA) $K_M$ and HA turnover number (TN) could be extrapolated from mannitol-dependent rate data in the micromolar concentration range. The low-affinity $K_M$ should have been extrapolated from rate data in the millimolar mannitol concentration range, but these data did not satisfy a single saturatable process. HPr, however, showed classic saturation behavior only at millimolar (low-affinity) mannitol concentrations. Consequently, we continue with the convention established by Lolkema et al. (1993) of reporting a HA-$K_M$ for mannitol and a HA-TN, both obtained at micromolar mannitol concentrations, and a low-affinity (LA) $K_M$ for HPr and a LA-TN obtained from rate data at millimolar mannitol concentrations. The kinetic measurements were done with solubilized vesicles of E. coli LGS-322 expressing overproducing constructs of mutant and wild-type EII\textsuperscript{mtl}. In addition, binding constants of mannitol to the nonphosphorylated enzyme were determined via flow dialysis (Lolkema et al., 1990). Table 1 shows these kinetic parameters for wild-type EII\textsuperscript{mtl} and all mutant EII\textsuperscript{mtl}’s. $K_D$ values differ by only a factor of 2--2.5 between the wild-type protein and all of the mutant proteins; the same is true for the LA-$K_M$ and the HA-$K_M$ except for C384W which is inactive due to the mutation at the phosphorylation site, C384. The TN’s for both the high-affinity and the low-affinity regimes differ by maximally a factor of 5, with the most drastic change coming from the replacement of W42 by phenylalanine. When all three other natural tryptophans are replaced by phenylalanines (FWFF), the TN’s are virtually unchanged. These data show that W42 is the most critical of the tryptophans but that none of the four tryptophans or C320 are essential for mannitol binding or phosphorylation.

Fluorescence Spectra of Isolated EII\textsuperscript{mtl}(Trp−). Wild-type EII\textsuperscript{mtl} and the various mutant EII\textsuperscript{mtl}’s were isolated by extraction of membrane vesicles with DOC followed by chromatography, first over hexyl-agarose, during which the detergent was changed to a poly(ethylene glycol)-based detergent, and finally over Q-Sepharose.

Initial experiments had shown that the enzyme was active and stable in C\textsubscript{10}E\textsubscript{6}, one of the components of decyl-PEG. Although this detergent also contained fluorescent impurities, an efficient route was found to remove these impurities via crystallization from hexane/isooctane (see Experimental Procedures). Figure 1 presents the emission spectrum of EII\textsuperscript{mtl}(Trp−), isolated by using the purified C\textsubscript{10}E\textsubscript{6}. Upon excitation at 290 nm, a typical tyrosine spectrum was obtained with a maximum at 305 nm, representing the 11 tyrosines present in EII\textsuperscript{mtl}(Trp−). The six single tryptophan mutants of EII\textsuperscript{mtl} examined in the remainder of this study have been purified using the same detergent and precautions (see Experimental Procedures).

Fluorescence Spectra of EII\textsuperscript{mtl} and EII\textsuperscript{mtl} Mutants. Figure 2 shows the corrected fluorescence emission spectra of wild-type EII\textsuperscript{mtl} and the single tryptophan mutant proteins, at $\lambda_{ex} = 290$ nm, before and after the addition of 3.4 μM mannitol. The data from these spectra are listed in Table 2. For the best estimates of the individual intensities and fluorescence emission maxima, it was necessary to correct for the contribution of the tyrosine residues to the spectra, as was done in the last three columns of the table. The most striking features of the spectra are the high intensity of 1.4 for EII\textsuperscript{mtl}, (Trp−)C320W and the very low intensity of 0.002 for EII\textsuperscript{mtl}, (Trp−)C384W relative to an intensity of 1, by definition, for wild-type EII\textsuperscript{mtl} which possesses four tryptophans. The other tryptophans also show different fluorescence intensities and emission wavelengths, but these are less extreme. The corrected emission maxima of the single tryptophan mutants vary from 324 nm for the tryptophan at position 30 to 340 nm for the tryptophan at position 109. These differences in

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Table 1: Mannitol Binding Constants and Kinetic Parameters for P-Enolpyruvate-Dependent Mannitol Phosphorylation Catalyzed by EII\textsuperscript{mtl} and Different Mutants of EII\textsuperscript{mtl}.

<table>
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<th>$K_D$ (nM)</th>
<th>$V_{\text{max}}$ (μM)</th>
<th>$K_M$ (μM)</th>
<th>$V_{\text{max}}$ (μM)</th>
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<tr>
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<td>4.5</td>
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<td>1315</td>
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<tr>
<td>EII\textsuperscript{mtl}(Trp−)C384W</td>
<td>126</td>
<td>X</td>
<td>X</td>
<td>X</td>
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*The binding experiments were carried out on solubilized vesicles of LGS-322 containing the different EII\textsuperscript{mtl} mutants or the wild-type enzyme. Vesicles were solubilized by diluting in 25 mM Tris-HCl, pH 7.6, 5 mM DTT, 5 mM MgSO\textsubscript{4}, and 0.25% (v/v) decyl-PEG followed by 5 min incubation at 25°C prior to the experiments. Mannitol binding at different mannitol concentrations (100, 200, 400, and 800 nM) was measured by flow dialysis at 25°C by a stepwise addition of mannitol from a 10 μM \textsuperscript{3}H-mtl stock in water. The mannitol binding constants were determined from the slope in a Scatchard plot. The total number of binding sites in the different experiments varied from 135 to 260 nM. The phosphorylation assay mixture used in the HPr-dependent mannitol assay, in which LA-$K_M$ and LA-TN of the different enzymes were determined, contained 25 mM Tris-HCl, pH 7.6, 5 mM DTT, 5 mM MgSO\textsubscript{4}, 5 mM PEP, 0.25% (v/v) decyl-PEG, 0.15 μM E, and 1 mM [\textsuperscript{14}C]mannitol. The EII\textsuperscript{mtl} and mutant concentrations varied from 3.8 to 16.4 nM (based on mannitol binding assays). The HPr concentrations were 1.3, 2.6, 5.2, 10.4, 20.8, and 41.6 μM. For the determination of LA-$K_M$ and LA-TN, a mixture containing 25 mM Tris-HCl, pH 7.6, 5 mM DTT, 5 mM MgSO\textsubscript{4}, 5 mM PEP, 0.25% (v/v) decyl-PEG, 0.15 μM E, and 4.8 μM HPr was used. The mannitol phosphorylation activity at 30°C was measured at \textsuperscript{3}H-mtl concentrations of 1.25, 2.5, 5, 10, and 20 μM on vesicles diluted to final EII\textsuperscript{mtl} (or mutant) concentrations varying from 96 to 205 PM. The activity measurements were repeated at least 2 times, leading to averaged values for the kinetic parameters. The variation in the results was always below 20%. The X’s for EII\textsuperscript{mtl}(Trp−)C384W mean no measurable activity.
Fluorescence of Single Tryptophan Mutants of EII<sub>mtl</sub>

**DISCUSSION**

Fluorescence spectroscopy can provide structural and dynamic data on biological macromolecules. In the case of membrane proteins, many studies have been done using fluorescent probes with excitation wavelengths $>340$ nm (Flitsch & Khorana, 1989; Shahrokh et al., 1991; Weimbs & Stoffel, 1992; Corbalan-Garcia et al., 1993; Jung et al., 1993; Valenzuela et al., 1994). Site-specific labeling which, preferably, does not introduce conformational changes is a prerequisite for their use. These demands are often difficult to satisfy because of the bulky and hydrophobic nature of most fluorescent labels. For this reason, numerous water-soluble proteins have been studied through their tryptophans which are excellent fluorescent probes of structure and dynamics. Interpretation of the fluorescence data is especially straightforward if only one tryptophan residue is present, a feature which can nowadays be realized routinely using site-directed mutagenesis. We have constructed six single tryptophan mutants which have enabled us to derive site-specific information in this study. The construction of a tryptophan-minus mutant of EII<sub>mtl</sub> was important because it enabled us to eliminate artifacts from fluorescence impurities present in the detergents and arising during purification.

**Fluorescent Impurities.** The spectrum of the tryptophan-minus protein should have shown a maximum between 300 and 305 nm due to tyrosine fluorescence from the 11 tyrosines in EII<sub>mtl</sub>. However, such a spectrum was only derived if the enzyme was isolated using a very pure detergent and all contact with plastics and rubber was avoided.

Our experience with the purification of EII<sub>mtl</sub>(Trp−) clearly indicates that without the above precautions isolated membrane proteins will be contaminated with fluorescent impurities (Robillard et al., 1996). This implies that many membrane proteins which have been isolated using these types of detergents may contain similar impurities. Some observations made in fluorescence studies on other membrane proteins either in detergents or reconstituted in phospholipids support this suggestion. In some cases, fluorescent emissions were monitored which could not have been due to the native fluorescence of the protein itself, like the shoulder near 415 nm in the emission spectrum of human erythrocyte hexose transport protein, which was ascribed to impurities in the lipid mixtures used (Pawagi & Deber, 1990). Of special interest are the studies on tryptophan-lacking enzymes, like...
the tryptophan-free E. coli lac permease where fluorescence emissions in the tryptophan region were due to impurities in the lipids used for reconstitution of the enzyme (Menezes et al., 1990). Although the fluorescence emission was explained as a contamination with tryptophan-containing proteins in the lipid preparation, it is possible that fluorescent impurities from detergent also contributed to this emission. The fluorescence of tryptophan residues in proteins purified with impure detergents like commercially available preparations of octyl glucoside, hydrogenated Triton X-100, and thesiti (Gorga & Lienhard, 1982; Ladokhin et al., 1991; Viguera et al., 1992; Pap et al., 1993) will overlap the fluorescence spectra of the impurities. Therefore, the contribution of the impurity to the overall fluorescence will go unnoticed. These impurities may lead to artifacts in interpretation of spectroscopic data and may also influence the crystallization properties of the protein.

Fluorescence Features of EII\textsuperscript{mtl}. The single tryptophan mutants of EII\textsuperscript{mtl} are all still able to bind mannitol and, with the exception of EII\textsuperscript{mtl}(Trp\textsuperscript{-})C384W, transport and phosphorylate the substrate. The mutated residues are not essential to the function of the enzyme, though some kinetic characteristics are slightly changed especially when W42 is replaced by phenylalanine. We can, therefore, conclude that no major changes in protein structure are induced by the mutations, and that the spectral data are relevant to the study of the wild-type EII\textsuperscript{mtl}. All of the positions investigated in the C domain are somewhat hydrophobic as indicated by the range of their emission maxima (324–340 nm). Within this range, a discrimination between more and less hydrophobic residue positions is possible. The schematic of the folding of the C domain in the membrane (Figure 5) as derived from fusion studies (Sugiyama et al., 1991) shows residues 30, 42, and 320 situated in transmembrane helices.
and 109 and 117 in a loop on the cytoplasmic side of the membrane. Three of the four natural tryptophan positions can be marked as less hydrophobic on the basis of their emission maxima: positions 42 (337 nm), 109 (340 nm), and 117 (339 nm). They could still be situated within the confines of the membrane bilayer but experience more polar surroundings due to other neighboring polar side chains. In contrast, the tryptophan at position 30 is extremely blue-shifted; its 324 nm emission maximum is close to that of tryptophan in hexane (320 nm), suggesting a hydrophobic surrounding. The relative intensities of the natural tryptophan positions are in accord with the expected values based on their hydrophobic nature; the more hydrophobic the environment, the higher the relative intensity of the observed fluorescence. The results found with I− quenching of the different mutants support the emission maxima data. Only EII mtl (WFFF) is very poorly quenched by I− (KSV 0.45 M−1), indicating an inaccessible location for W30. Positions 42 and 320 seem to be somewhat more exposed (KSV 1.13 M−1 and 1.18 M−1, respectively), whereas positions 109 and 117 are more easily quenched (KSV 1.75 M−1 and 1.84 M−1, respectively), indicating an accessible location. At first glance, the difference between W30 and W42 is somewhat surprising, both being schematically situated in the membrane and still being different in the hydrophobicity of their surroundings. Stowell and Rees (1995) have correlated the 3D structural data so far collected for membrane proteins and find consistently that tryptophans are often situated at the ends of membrane-spanning helices as though they were anchoring the helix in the membrane. However, the exact position and surroundings of the indole ring differed considerably. In some cases, the hydrophobic side of the ring faced into the membrane while the polar side, with its indole nitrogen, faced the surface and was H-bonded to other polar groups. In other cases, the surrounding was entirely hydrophobic. Such observations could account for the differences between W30 and W42. The emission maxima and relative emission intensities found for the different mutants, as well as the Stern−Volmer quenching constants, are in line with the expectations based on the topology of EII mtl presented in Figure 5 (Sugiyama et al., 1991).
Residues 30, 42, and 320 are probably in membrane-spanning α-helices, whereas residues 109 and 117 are in a more hydrophilic environment which could be a cytoplasmic loop or a polar channel in the membrane-spanning structure.

Binding of mannitol to EIImtl leads to an increased fluorescence, which is mainly due to an increased intensity of W30 as can be seen in Figure 4. This observation plus the influence of the replacement of W42 on the HA- and LA-TN (Table 1) is evidence that the first putative helix of EIImtl (Sugiyama et al., 1991) is somehow involved in the mannitol binding and phosphorylation steps. Recently, similar increases in fluorescence intensity have been observed upon carbohydrate binding in another class of transporters, the E. coli melibiose permease (Mus-Veteau et al., 1995) and E. coli lactose permease (Weitzman et al., 1995).

The two mutants with a tryptophan at nonnatural positions, 320 and 384, have emission maxima which also support the predicted topology but, more interestingly, are extreme with respect to their emission intensities. We have no explanation for the unusually high relative intensity for the tryptophan at position 320, which is predicted to be in the middle of a membrane-spanning helix. The unusually low intensity of W384 in the cytoplasmic B domain most likely results from quenching by deprotonated residues in the proximity of this residue. The unusually low intensity of W384 in the cytoplasmic B domain most likely results from deprotonated residues in the proximity of this residue.

In conclusion, various fluorescence experiments with single Trp mutants of EIImtl have resulted for the first time in spectroscopic data supporting the proposed 2D model of EIImtl based on fusion experiments.

REFERENCES


