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

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ABSTRACT: The fluorescence properties of six different single Trp mutants of the mannitol-specific transporter of Escherichia coli, enzyme II$_{mtl}$ (EII$_{mtl}$),† 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 phenylalanine. 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 EII$_{mtl}$ was created to develop methods to remove or avoid the impurities. The exercise was judged successful only when an EII$_{mtl}$(Trp$^-$) fluorescence spectrum could be routinely obtained showing characteristic tyrosine fluorescence and no significant fluorescence in the tryptophan region.

The mannitol-specific transport protein of Escherichia coli, enzyme II$_{mtl}$ (EII$_{mtl}$),† is a member of the P-enolpyruvate-dependent phosphotransferase 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 phenylalanine. 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 EII$_{mtl}$ was created to develop methods to remove or avoid the impurities. The exercise was judged successful only when an EII$_{mtl}$(Trp$^-$) fluorescence spectrum could be routinely obtained showing characteristic tyrosine fluorescence and no significant fluorescence in the tryptophan region.  

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Abbreviations: mtl, mannitol; HPr, histidine-containing protein; EI, enzyme I of the phosphoenolpyruvate-dependent carbohydrate transport system; EI$^{II_{mtl}}$, mannitol-specific transport protein and phosphorylating enzyme; EI$^{II_{mtl}}$(Trp$^-$), tryptophan-minus mutant of EI$^{II_{mtl}}$; EI$^{II_{mtl}}$(WFFT), EI$^{II_{mtl}}$(FPFW), EI$^{II_{mtl}}$(FFFW), and EI$^{II_{mtl}}$(FFFW), EI$^{II_{mtl}}$ mutants with tryptophan 30, 42, 109, and 117 each present as a single tryptophan, respectively, while the other three tryptophans had been replaced by phenylalanine; EI$^{II_{mtl}}$(Trp$^-$)(C320W), EI$^{II_{mtl}}$ mutant containing a tryptophan only at position 320; EI$^{II_{mtl}}$(Trp$^-$)(C384W), EI$^{II_{mtl}}$ mutant containing a tryptophan only at position 384; DTT, dithiothreitol; GSH, reduced glutathione; DOC, sodium deoxycholate; decyl-PEG, decylpoly(ethylene glycol) 300; PEP, phosphoenolpyruvate; C$_{6}$H$_{5}$, decylpentadecylpentadecyl; cmc, critical micelle concentration; TN, turnover number; K$_{SV}$, Stern–Volmer quenching constant.
Fluorescence of Single Tryptophan Mutants of EII<sup>mtl</sup>

**EXPERIMENTAL PROCEDURES**

**Materials.** Sodium deoxycholate (DOC) was obtained from Sigma. Decylpenta(ethylene glycol) (C<sub>10</sub>E<sub>5</sub>) and decylpoly(ethylene glycol) 300 (decy-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. ∆-[<sup>1-14</sup>C]Mannitol (59 mCi/mmol; 1 mCi = 37 MBq) was purchased from the Radiochemical Center Amersham; ∆-[<sup>1-3</sup>H(N)]mannitol (976.8 GBq/mmol) was obtained from DuPont NEN Research Products. Hexane was distilled from P<sub>2</sub>O<sub>5</sub>. 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<sup>thi-1</sup>, hisG1, argG6, metB1, tonA2, supE44, rpsL104, lacY1, galT6, galR49, galA50, ∆(mtla<sub>p</sub>), mtld<sub>+</sub>, ∆(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<sup>mtl</sup> as well as for the wild-type EII<sup>mtl</sup>. 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′ GTG CGG AAA CCC TGT TGG 3′; W109F, 5′ CTT AAT GCA AAA GCC CCC CAG 3′; and W117F, 5′ ACC GTC TAC AAA GCG GTC GAA 3′.

Two single tryptophan mutants, EII<sup>mtl</sup>(Trp<sup>−</sup>)C320W and EII<sup>mtl</sup>(Trp<sup>−</sup>)C384W, were created in the tryptophan-minus construct. The primers used were EII<sup>mtl</sup>(Trp<sup>−</sup>)C384W, 5′ CC GCC GTC CCA GGC AAC GAT G 3′; and EII<sup>mtl</sup>(Trp<sup>−</sup>)C320W, 5′ AT CGC CGG CCA GAC ACC CG 3′.

All constructs were completely sequenced and, except for the intended change and a silent mutation in the codon of glycine 289 (GGC) in the EII<sup>mtl</sup>(WFFF) mutant, were found to agree with the wild-type sequence (Lee & Saier, 1983).

**Purification of Detergent.** C<sub>10</sub>E<sub>5</sub> [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/H<sub>2</sub>O 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 C<sub>10</sub>E<sub>5</sub>. If necessary, they were pretreated for several days with analytical grade methanol and C<sub>10</sub>E<sub>5</sub>-containing buffer until virtually no fluorescent material was present. Resins were always prewashed with C<sub>10</sub>E<sub>5</sub>-containing buffers until no fluorescence was detected in the elution buffer. Servamp 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 Na<sub>2</sub>-EDTA to remove ultraviolet-adsorbing materials and metal ions (Reynolds et al., 1967). Prior to the dialysis of samples in purified C<sub>10</sub>E<sub>5</sub>, the dialysis tubing was further treated by storage for at least 24 h in C<sub>10</sub>E<sub>5</sub>-containing buffer.

**Protein Purification.** EII<sup>mtl</sup>(Trp<sup>−</sup>) and all single tryptophan proteins were purified essentially as described for EII<sup>mtl</sup>-C384S (Robillard et al., 1993). The only modification of 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 C<sub>10</sub>E<sub>5</sub> led to altered binding characteristics of EII<sup>mtl</sup> 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) C<sub>10</sub>E<sub>5</sub>. The elution was also performed at room temperature using a gradient of 0.15–4% (v/v) C<sub>10</sub>E<sub>5</sub> in the same wash buffer. The fractions containing the enzyme, which eluted at about 2.5% (v/v) C<sub>10</sub>E<sub>5</sub>, were dialuted 5 times with 20 mM Tris-HCl, 1 mM DTT, and 0.25% C<sub>10</sub>E<sub>5</sub>, 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) C<sub>10</sub>E<sub>5</sub>. 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<sup>mtl</sup> and the different mutants was measured as described (Robillard & Blaauw, 1987). The assay mixture contained 25 mM Tris, pH 7.6, 5 mM MgCl<sub>2</sub>, 5 mM DTT, 5 mM PEP, 0.25% (v/v) decyl-PEG, 10 µM HPr, 0.2 µM EI, and 0.5 mM [<sup>14</sup>C]-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<sup>mtl</sup> and EII<sup>mtl</sup> Mutants.** The concentration of EII<sup>mtl</sup> 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 (K<sub>b</sub> ~ 100 nM) per EII<sup>mtl</sup> 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) C<sub>10</sub>E<sub>5</sub>, 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 MgCl<sub>2</sub>, 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<sup>mtl</sup> and mutants of EII<sup>mtl</sup>.

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 $K_M$\textsuperscript{mtl}. The high-affinity (HA) $K_M$\textsuperscript{mtl} and HA turnover number (TN) could be extrapolated from mannitol-dependent rate data in the micromolar concentration range. The low-affinity $K_M$\textsuperscript{mtl} should have been extrapolated from rate data in the millimolar mannitol concentration range, but these data did not satisfy a single saturable 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-$K_M$\textsuperscript{mtl} for mannitol and a HA-TN, both obtained at micromolar mannitol concentrations, and a low-affinity (LA) $K_M$\textsuperscript{HPr} for HPr and a LA-TN obtained from rate data in 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}\textsuperscript{a}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-$K_M$\textsuperscript{HPr} and the HA-$K_M$\textsuperscript{mtl} 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\textsuperscript{−}). Wild-type EII\textsuperscript{mtl} and the various mutant EII\textsuperscript{mtl}\textsuperscript{a}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.

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}\textsuperscript{a}

<table>
<thead>
<tr>
<th>EII\textsuperscript{mtl}</th>
<th>$K_D$ (nM)</th>
<th>LA-$K_M$\textsuperscript{HPr} (µM)</th>
<th>LA-TN (min\textsuperscript{−1})</th>
<th>HA-$K_M$\textsuperscript{mtl} (µM)</th>
<th>HA-TN (min\textsuperscript{−1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>EII\textsuperscript{mtl}</td>
<td>56</td>
<td>8.0</td>
<td>4114</td>
<td>8.0</td>
<td>1481</td>
</tr>
<tr>
<td>EII\textsuperscript{mtl}(Trp\textsuperscript{−})</td>
<td>40</td>
<td>4.0</td>
<td>992</td>
<td>2.6</td>
<td>428</td>
</tr>
<tr>
<td>EII\textsuperscript{mtl}(WFFF)</td>
<td>105</td>
<td>5.6</td>
<td>2053</td>
<td>4.0</td>
<td>976</td>
</tr>
<tr>
<td>EII\textsuperscript{mtl}(FWFF)</td>
<td>49</td>
<td>7.7</td>
<td>5139</td>
<td>5.9</td>
<td>1129</td>
</tr>
<tr>
<td>EII\textsuperscript{mtl}(FFWF)</td>
<td>74</td>
<td>3.1</td>
<td>1552</td>
<td>2.6</td>
<td>835</td>
</tr>
<tr>
<td>EII\textsuperscript{mtl}(FFFW)</td>
<td>45</td>
<td>4.0</td>
<td>1374</td>
<td>2.9</td>
<td>705</td>
</tr>
<tr>
<td>EII\textsuperscript{mtl}(Trp\textsuperscript{−})C320W</td>
<td>41</td>
<td>4.2</td>
<td>1315</td>
<td>2.9</td>
<td>599</td>
</tr>
<tr>
<td>EII\textsuperscript{mtl}(Trp\textsuperscript{−})C384W</td>
<td>126</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
</tbody>
</table>

\textsuperscript{a} 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 1\textsuperscript{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 phosphorylation assay, in which LA-$K_M$\textsuperscript{HPr} 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 EL, 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-$K_M$\textsuperscript{mtl} and HA-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 EL, and 4.8 µM HPr was used. The mannitol phosphorylation activity at 30 °C was measured at 1\textsuperscript{H}-mtl concentrations of 1.25, 2.5, 5, 10, and 20 nM 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\textsuperscript{−})C384W mean no measurable activity.
the emission maxima for the individual tryptophans are indicative of different environments within the C domain.

Titrations of the different single tryptophan mutants with I− (Figure 3) indicate that the location of W30 is not accessible because it is poorly quenched (κSV 0.45 M−1), while tryptophan residues 109 and 117 are more accessible (κSV 1.75 and 1.84 M−1, respectively). The quenching constants for tryptophans at positions 42 and 320 (1.13 M−1 and 1.18 M−1, respectively) suggest a partly buried position, more accessible than position 30 but more buried than positions 109 and 117.

The tryptophan at position 384 in the cytoplasmic B domain of the protein is not only special with respect to its low relative intensity, but also shows the most red-shifted emission maximum at 346 nm; this is close to the value of 348 nm for completely water-exposed tryptophan (Teale & Weber, 1957).

Effect of Mannitol Binding and Phosphorylation. There is a clear increase (+8% between 300 and 400 nm) in the fluorescence intensity of wild-type EIImut upon addition of 3.4 µM mannitol (Figure 2A), which is accounted for almost entirely by the increase in intensity of W30 (+13%) [Figure 2B, EIImut(WFFF)]. This indicates that a change in this region of the protein is induced by mannitol binding. Spectra of EIImut(WFFF) taken as a function of mannitol concentration were used to estimate the mannitol binding constant. A best fit yielded a value of 129 nM, close to the value of 105 nM determined by flow dialysis (Table 1). Phosphorylation of the enzyme did not alter the fluorescence spectrum of EIImut, indicating that none of the four natural tryptophan residues experiences large structural changes upon phosphorylation.

The tryptophan in the C384W mutant, located in the B domain of the enzyme, has an abnormally low intensity (Figure 2D). The B domain possesses a carboxylate and a tyrosine in the region of the C384 phosphorylation site (AB et al., unpublished data). The tyrosine, which is the only one in the B domain, has been shown to be ionized at neutral pH (Meijberg et al., 1996). Either residue could function as an efficient quencher of W384 and account for the anomalously low relative intensity. Figure 4 shows that lowering the pH gives rise to an increased fluorescence emission intensity. This could be due to protonation of the tyrosinate and/or the carboxylate group; however, a pH-dependent structural change or denaturation of IIBmut would also reduce the quenching by disrupting the tertiary structure responsible for the tryptophan−tyrosinate or tryptophan−carboxylate interaction. Unfolding has indeed been observed; circular dichroism spectra of IIBmut measured at various pHs demonstrate that IIBmut unfolds at pH values lower than 6 (Meijberg et al., 1996).

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 (Fitsch & 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 EIImut 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 EIImut. 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 EIImut(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

![Figure 1: Corrected fluorescence emission spectrum of EIImut (Trp−) purified with crystallized C10E5. The sample was dialyzed for 3 h against 20 mM Tris-HCl, 100 mM NaCl, 0.1% C10E5, and 1 mM GSH to remove absorbing molecules like oxidized DTT, leading to absorptions below 0.05 in the wavelength interval of interest. The spectrum was recorded at 5 °C with excitation at 290 nm using bandwidths of 4 nm on both monochromators. The enzyme concentration was 0.3 µM.](image-url)
the trypotphan-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 thesits (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

### Table 2: Steady-State Fluorescence of EII\textsuperscript{mtl} and Mutants of EII\textsuperscript{mtl} \(^a\)

<table>
<thead>
<tr>
<th>type</th>
<th>(\lambda_{\text{max}}) (nm)</th>
<th>(\lambda_{\text{max}}) (nm)</th>
<th>relative intensity</th>
<th>(\lambda_{\text{max}}) (nm) + mtl</th>
</tr>
</thead>
<tbody>
<tr>
<td>EII\textsuperscript{mtl}</td>
<td>330</td>
<td>331</td>
<td>1 (def.)</td>
<td>331</td>
</tr>
<tr>
<td>EII\textsuperscript{mtl} (Trp\textsuperscript{-})</td>
<td>305</td>
<td>X</td>
<td>X</td>
<td>X</td>
</tr>
<tr>
<td>EII\textsuperscript{mtl} (WFFF)</td>
<td>316.5</td>
<td>324</td>
<td>0.296</td>
<td>322</td>
</tr>
<tr>
<td>EII\textsuperscript{mtl} (FWFF)</td>
<td>330.5</td>
<td>337</td>
<td>0.121</td>
<td>336</td>
</tr>
<tr>
<td>EII\textsuperscript{mtl} (FFWF)</td>
<td>328</td>
<td>340</td>
<td>0.084</td>
<td>338</td>
</tr>
<tr>
<td>EII\textsuperscript{mtl} (FFFW)</td>
<td>332</td>
<td>339</td>
<td>0.118</td>
<td>337</td>
</tr>
<tr>
<td>EII\textsuperscript{mtl} (Trp\textsuperscript{-}) C320W</td>
<td>329</td>
<td>330</td>
<td>1.390</td>
<td>329</td>
</tr>
<tr>
<td>EII\textsuperscript{mtl} (Trp\textsuperscript{-}) C384W</td>
<td>307</td>
<td>346</td>
<td>0.002</td>
<td>345</td>
</tr>
</tbody>
</table>

\(^a\) The data were collected from corrected steady-state fluorescence emission spectra, recorded at 5 \(^\circ\)C with an excitation wavelength of 290 nm. The samples were dialyzed and diluted to 0.3 \(\mu\)M enzyme if necessary as described under the Experimental Procedures. The relative intensities were based on the areas under the corrected emission spectra between 300 and 400 nm. The integrated area of EII\textsuperscript{mtl} was defined as having an intensity of 1. In the last three columns, the EII\textsuperscript{mtl} (Trp\textsuperscript{-}) spectrum was subtracted from the spectra in order to correct for the tyrosine contribution.
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

![Figure 3: Stern−Volmer plots for quenching of the different single tryptophan mutants of EII mtl with I−. F0 is the integrated fluorescent emission without quencher; FKI is the integrated emission at different concentrations of KI. The fluorescence emission was measured at 5 °C, on 0.3 µM enzyme solutions, with excitation at 290 nm. KI was added from a fresh 3 M stock solution in water also containing a trace of Na2S2O3 to prevent the formation of I3−. The [I−] shown are 0, 133, 213, and 300 mM for EII mtl (WFFF), EII mtl (FWFF), and EII mtl (Trp−C320W) and 0, 133, 178, 213, and 300 mM for EII mtl (FFWF) and EII mtl (FFFW), yielding a decreased intensity for all mutants. Aspecific effects due to the increased ionic strength were prevented by using two stock solutions during the titration: one containing 300 mM KI, the other containing 300 mM KCl. The lines for EII mtl (FFWF) and EII mtl (FFFW) were calculated using data points only up to 213 mM KI; static quenching occurred in these mutants at higher KI concentrations, resulting in upward curvature.](image3)

![Figure 4: pH dependence of the fluorescence of EII mtl (Trp−)-C384W. The solid line represents the corrected emission spectrum of EII mtl (Trp−)-C384W at pH 7.92; the broken line represents the corrected emission spectrum at pH 3.45. Spectra were recorded at 5 °C on a sample containing 0.3 µM EII mtl (Trp−)-C384W in 20 mM Tris-HCl, 100 mM NaCl, 1 mM GSH, and 0.25% C10E5, pH 7.92, with excitation at 290 nm. The pH was lowered by stepwise addition of small volumes of diluted HCl to 3.45. No change in absorption was seen during this pH change, indicating that no precipitate was formed.](image4)

![Figure 5: A 2D representation of the membrane disposition of the C domain of EII mtl as proposed by Sugiyama et al. (1991) on the basis of fusion studies. The C domain residue positions investigated in this study, 30, 42, 109, 117, and 320, and position 384 in the B domain are enclosed in boxes.](image5)

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 EII<sup>mt</sup> 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 EII<sup>mt</sup> (Sugiyama et al., 1991) is somehow involved in the mannitol binding and phosphorylation steps. Recently, similar increases in fluorescence intensity have been observed upon carbohydate 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 tryptophan. Whether these residues and their deprotonated state are critical for the mechanism of phosphoryl group transfer to mannitol is currently being investigated.

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

REFERENCES


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