Substrate-induced Conformational Changes in the Membrane-embedded IIC\textsubscript{mtl}-domain of the Mannitol Permease from \textit{Escherichia coli}, EnzymeII\textsubscript{mtl}, Probed by Tryptophan Phosphorescence Spectroscopy* \\
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Membrane-bound transport proteins are expected to proceed via different conformational states during the translocation of a solute across the membrane. Tryptophan phosphorescence spectroscopy is one of the most sensitive methods used for detecting conformational changes in proteins. We employed this technique to study substrate-induced conformational changes in the mannitol permease, EnzymeIImtl, of the phosphoenolpyruvate-dependent phosphotransferase system from \textit{Escherichia coli}. Ten mutants containing a single tryptophan were engineered in the membrane-embedded IIC\textsubscript{mtl}-domain, harboring the mannitol translocation pathway. The mutants were characterized with respect to steady-state and time-resolved phosphorescence, yielding detailed, site-specific information of the Trp microenvironment and protein conformational homogeneity. The study revealed that the Trp environments vary from apolar, unstructured, and flexible sites to buried, highly homogeneous, rigid peptide cores. The most remarkable example of the latter was observed for position 97, because its long sub-second phosphorescence lifetime and highly structured spectra in both glassy and fluid media imply a well-defined and rigid core around the probe that is typical of \beta-sheet-rich structural motifs. The addition of mannitol had a large impact on most of the Trp positions studied. In the case of position 97, mannitol binding induced partial unfolding of the rigid protein core. On the contrary, for residue positions 126, 133, and 147, both steady-state and time-resolved data showed that mannitol binding induces a more ordered and homogeneous structure around these residues. The observations are discussed in context of the current mechanistic and structural model of EII\textsubscript{mtl}.

The mannitol permease from the Gram-negative bacterium \textit{Escherichia coli}, EnzymeII\textsubscript{mtl} (EII\textsubscript{mtl})\textsuperscript{1,2,3} (1, 2), is responsible for the uptake and consecutive phosphorylation of mannitol (reviewed in Ref. 1). EII\textsubscript{mtl} consists of three covalently linked domains (from N to C termini): a membrane-embedded IIC\textsubscript{mtl}-domain harboring the mannitol-translocation pathway (2), and two cytosolic domains (IIB\textsubscript{mtl} and IIA\textsubscript{mtl}) responsible for phosphoryl transfer. EII\textsubscript{mtl} becomes phosphorylated via a cascade of phosphoryl group-transfer reactions, starting with the hydrolysis of phosphoenolpyruvate by the cytosolic kinase EnzymeI (EI). The phosphate moiety from phosphorylated EI is transferred to HPr, a small cytosolic protein. Subsequently, His-554 in the IIA\textsubscript{mtl}-domain is phosphorylated by P-HPr, and transfers the phosphate to Cys-384 in the IIB\textsubscript{mtl}-domain. The phosphate is then donated to mannitol bound at the IIC\textsubscript{mtl}-domain, resulting in the release of mannitol 1-phosphate in the cytoplasm. Phosphorylation of EII\textsubscript{mtl} activates the carrier, resulting in a two-to-three orders of magnitude increase in the transport rate (3–5).

A \textit{phoA} fusion study and hydrophathy analysis of the IIC\textsubscript{mtl}-domain resulted in a topology model with three small periplasmic loops, two large cytoplasmic loops, and six putative membrane-spanning helices (6). It has been proposed that both large cytoplasmic loops fold back into the membrane-embedded part of the protein, lining up a hydrophilic pathway for the translocation of the carbohydrate (7). New structural insight on the basis of cysteine-scanning mutagenesis in the first proposed cytoplasmic loop provided evidence for the presence of this loop protruding, at least partly, into the bilayer (8). For the subcloned IIC\textsubscript{mtl} domain, a two-dimensional projection structure at 5-Å resolution was determined by electron microscopy crystallography (9). Six regions of high density were found, possibly reflecting six membrane-spanning helices.

Of the available spectroscopic techniques, Trp phosphorescence spectroscopy is one of the most sensitive approaches used to study changes in protein conformation, due to the extremely slow (radiative) de-excitation rate of the triplet excited state (~0.2 s\textsuperscript{-1}). This makes Trp phosphorescence 10\textsuperscript{8} times more sensitive for quenching processes than Trp fluorescence. A conformational change, nearby or more remote from the Trp probe, is expected to induce a different quenching pattern on this time scale, and thus a change in the phosphorescence lifetime ($\tau_P$). The observation of multiple $\tau_P$ values for a single Trp position reflects the presence of different protein conformational states, which do not rapidly interchange on the time scale of $\tau_P$. The ability to quench the Trp triplet state is governed by the local viscosity ($\eta_L$), and the relation between $\tau_P$ and $\eta_L$ is well established (10, 11). The information obtained by measuring $\tau_P$ combined with the recording of emission

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4 The abbreviations used are: EII\textsubscript{mtl}, EnzymeII\textsubscript{mtl} from \textit{E. coli}; $\lambda_{\alpha-D}$, peak wavelength of the 0.0- vibrational band in the phosphorescence spectrum; BW, bandwidth of the 0.0-vibrational band at 2/3-height; $\lambda_G$, centre of gravity of the phosphorescence spectrum; $\Delta(\tau_p)$, change in the centre of gravity upon thermal relaxation; $\tau_P$, phosphorescence lifetime; $\lambda_F$, fluorescence emission maximum; mtl, mannitol; PG, 1,2-propyleneglycol; C\textsubscript{10}E\textsubscript{5}, decylpentaethyleneglycol ether; decylPEG, decylpoly(ethyleneglycol)\textsubscript{300}.
5 Nomenclature of the enzymes: EII\textsubscript{mtl}, wild-type EnzymeII\textsubscript{mtl}; TL, EII\textsubscript{mtl} where the four native tryptophans of wild-type EII\textsubscript{mtl} (at positions 30, 42, 109, and 117) have been replaced with phenylalanines; Trp-66, Trp-97, Trp-114, Trp-126, Trp-133, Trp-147, Trp-167, Trp-188, and Trp-198 refer to single-Trp EII mutants based on TL; IIC-TL and IIC-\textsubscript{mtl}.

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spectra of the protein in both the glass-state and in the fluid state, provides site-specific, structural information about the Trp microviscosity (H9257), micropolarity, and protein conformational heterogeneity. Together with fluorescence, phosphorescence can distinguish whether a residue is placed in a superficial, mobile, and solvent-exposed location in the protein or when it is in a buried and rigid part.

The wild-type EIImtl protein has four Trp residues all located in the membrane-embedded IICmtl-domain. The fluorescence and phosphorescence characteristics of single-Trp mutants have pointed toward a large variation in polypeptide structure among the sites 30, 42, 109, and 117, as well a distinct response to the binding of mannitol and phosphorylation (12–16). The present study extends this approach with ten single-Trp mutants, containing tryptophans in either putative transmembrane helices or cytoplasmic loops of the IICmtl-domain (Fig. 1).

The construction of single-Trp mutants—The construction of the functional Trp-less (TL) EIImtl construct with a N-terminal His6 tag will be published elsewhere.6 In this construct all four native Trp residues at positions 30, 42, 109, and 117 were replaced with phenylalanines. The mutations resulting in the single-Trp mutants using TL as a basis (Trp-66, Trp-97, Trp-114, Trp-126, Trp-133, Trp-147, Trp-167, Trp-188, and Trp-198) were introduced using the QuikChange site-directed mutagenesis kit from Stratagene. In each mutant a phenylalanine was replaced with a Trp, except for Trp-66, where a tyrosine was mutated into a Trp. The sequences were confirmed by nucleotide sequence analysis. The IICmtl mutants were constructed by cutting the plasmids harboring the wild-type IICmtl-His6 (with a C-terminal His6 tag) (17), and TL and Trp-97 (EII constructs; see above), with restriction enzymes BbvC1 and Eco47III (at amino acid positions 15 and 237, respectively).

MATERIALS AND METHODS

Chemicals and Reagents—D-[1-3H]Mannitol (17.0 Ci/mmol, batch 3499-326) was purchased from PerkinElmer Life Sciences. D-[1-

6 E. P. P. Vos, manuscript in preparation.

FIGURE 1. Membrane topology model of the IICmtl-domain of EIImtl. The topology is based on the model presented by Vervoort et al. (8), using the algorithm TMHMM 2.0 (40) to determine the start and ending of transmembrane α-helices. Outside and Inside represent the peri- and cytoplasmic side of the membrane, respectively. The Greek capitals indicate the eight predicted transmembrane helices. The amino acid residues mutated into a tryptophan and used in this study are shown in black; the native tryptophans in the wild-type protein are shown in gray.

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The 665-bp fragment was isolated from the TL and Trp-97 mutants and ligated into the wild-type IIC<sup>mtl</sup>-His<sub>6</sub> plasmid, without this 665-bp fragment, yielding IIC-Trp-97, and IIC<sup>-</sup>-TL, respectively.

**Cell Growth, Isolation of Inside-out Membrane Vesicles, and Protein Purification**—The plasmids harboring the single-Trp-mutated mtl<sup>A</sup> genes (pMamtla<sub>H</sub>-, EIImtl-F→W or pMamtla<sub>M</sub>-, IICtl-F→W-His<sub>6</sub>) were transformed and subsequently grown in bacterial strain E. coli LGS322 (F<sup>−</sup> thi-1, hisG1, argG6, metB1, tonA2, supE44, rpsL104, lacY1, galT6, galR49, galA50, <i>Δ</i>(mtlA<sup>−</sup>i)<i>Δ</i>(mtlA<sup>−</sup>i)</i>), mtl<sup>DF</sup>, Δ(gutR<sup>−</sup>MBDA-reca)) as described before (18). Inside-out membrane vesicles were prepared by passage of the cells through a French Press at 10,000 p.s.i., essentially as described (19). The membrane vesicles were washed once in 25 mM Tris-HCl, pH 7.6, 5 mM dithiothreitol, 2 mM reduced glutathione, plus 0.25% C<sub>10</sub>E<sub>5</sub>, loaded onto Q-Sepharose, washed with 10 column volumes of the same buffer, and subsequently eluted in a single step using 4 column volumes of the above buffer, supplemented with 400 mM NaCl (Suprapur, Merck). All fluorescence and phosphorescence measurements were performed using this buffer.

All single-Trp EII<sup>mtl</sup> mutants were purified using Ni-NTA affinity chromatography as described (20). To remove all traces of the tryptophan phosphorescence quencher histidine (used to elute the protein from Ni-NTA) from the EII<sup>mtl</sup> mutant preparations, pooled Ni-NTA fractions were dialyzed five times in buffer (25 mM Tris-HCl, pH 7.6, 2 mM imidazole, plus 0.25% C<sub>10</sub>E<sub>5</sub>, 10 mM 2-mercaptoethanol, 1% (w/v) Brij<sub>35</sub>-D-maltopyranoside, plus 10 mM imidazole for 15 min at room temperature. After spinning down the non-solubilized material (10 min, 250,000 × g, 4 °C), the supernatant was mixed with washed Ni-NTA resin by stirring for 1 h at 4 °C. After draining the flow-through, the column was subsequently washed with 10 column volumes buffer A (25 mM Tris-HCl, pH 7.6, 200 mM NaCl, 0.35% decylPEG, 10 mM 2-mercaptoethanol, plus 10 mM imidazole), and 10 column volumes buffer B (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.25% decylPEG, plus 10 mM 2-mercaptoethanol). The His-tagged IIC<sup>mtl</sup> mutants were batch-wise eluted from Ni-NTA with 80 mM L-histidine (in 25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.25% decylPEG, plus 10 mM 2-mercaptoethanol). To remove the histidine, the same purification procedure as for the single-tryptophan EII<sup>mtl</sup> mutants was followed, using SF-Sepharose instead of Q-Sepharose.

For both the EII<sup>mtl</sup>- and IIC<sup>mtl</sup>- proteins, phosphorescence decay curves were both measured with pulsed excitation (λ<sub>ω</sub> = 288 nm) on a customized apparatus (24), modified to implement spectral measurements by means of a charge-coupled device camera. Pulsed excitation was provided by a frequency-doubled Nd/Yag-pumped dye laser (Quanta Systems, Milan, Italy) with pulse duration of 5 ns and a typical energy per pulse of 0.5–1 mJ. For spectra measurements the emission was collected at 90° from the excitation and dispersed by a 0.3-m focal length triple grating imaging spectrograph (SpectraPro-2300i, Acton Research Corp., Acton, MA) with a band pass ranging from 1.0 to 0.2 nm. The emission was monitored by a back-illuminated 1340×400-pixels charge-coupled device camera (Princeton Instruments, Spectroscopy-10400B(XTE), Roper Scientific Inc., Trenton, NJ) cooled to −60 °C. In low temperature glasses, the phosphorescence spectrum of Trp was overlapped by a relatively intense background from solvent impurities and tyrosinate, an emission that decayed to negligible levels during the initial 3–4 s from the excitation pulse. Background-free Trp spectra were obtained by opening the mechanical shutter controlling the emission to the spectrograph after a delay of 3 s. In fluid solutions, where the lifetime of Trp phosphorescence is much shorter than the 6 s in glassy media, spectra were recorded by integrating multiple excitation pulses at a repetition frequency up to 10 Hz. To block overlapping prompt fluorescence and short-lived background from the detector, laser excitation was synchronized to a fast mechanical chopper opening the emission slit 35 µs after the laser pulse. In general, even with the shortest-lived protein phosphorescence less than 100 pulses was sufficient to obtain satisfactory signal-to-noise ratios. Besides averaging multiple pulses, the signal-to-noise ratio of very weak signals, which are characterized by relatively broad spectra, was further improved by horizontal binning of channels (2–4 channels), with no effect on spectral resolution. Under these extreme conditions the background signal, represented by a broad band peaked around 500 nm, was not negligible and was subtracted from the total spectrum, by using the spectrum of a TL control.

Phosphorescence decays were monitored by collecting the emission at 90° from vertical excitation through a filter combination with a trans-
**RESULTS**

**Catalytic Properties of Single-Trp Mutants**

The Trp-less and single-Trp EII and IIC mutants were tested for mannitol binding, and the results are summarized in TABLE ONE. All mutants bound mannitol with high affinity in the nanomolar range, comparable to the wild-type protein (21), except Trp-198 ($K_D$, of 375 nM), Trp-97, and IIC-Trp-97, which showed a significant decreased binding affinity with $K_D$ values of $\sim$2 μM. The phosphorylation activities in intact membrane vesicles were more or less the same for all EII mutants and comparable to wild-type and TL (12), indicating the functionality of all mutants. Phosphorylation activity for the IIC mutants could not be measured, because they lack the IIBmtl-domains for phosphoryl-group transfer.

### Phosphorescence Spectroscopy on EIImtl

- The fluorescence emission maximum ($\lambda_{0,0}$) provides an estimate of the polarity of the Trp environment as $\lambda_F$ for the free chromophore increases from 300 nm in non-polar butanol to 350 nm in aqueous solutions (25). Only the emission of Trp-97 and IIC-Trp-97 showed a blue-shifted maximum ($\lambda_F$ = 318 nm). The other mutants displayed maxima of $>325$ nm. The emission maxima of Trp-97 and IIC-Trp-97 are similar to that of tryptophan in hexane (320 nm), suggesting a very hydrophobic, non-polar environment for these residues.

- **Fluorescence Spectra at Room Temperature**—The fluorescence emission maximum ($\lambda_{0,0}$) provides an estimate of the polarity of the Trp environment as $\lambda_F$ for the free chromophore increases from 300 nm in non-polar butanol to 350 nm in aqueous solutions (25). Only the emission of Trp-97 and IIC-Trp-97 showed a blue-shifted maximum ($\lambda_F$ = 318 nm). The other mutants displayed maxima of $>325$ nm. The emission maxima of Trp-97 and IIC-Trp-97 are similar to that of tryptophan in hexane (320 nm), suggesting a very hydrophobic, non-polar environment for these residues.

- **Phosphorescence Spectra in Glasses at 140 K**—In a rigid medium, as a low temperature glass, the spectrum of Trp displays a pronounced vibronic structure with a well resolved 0,0-vibrational band. Although the wavelength of the 0,0-vibrational band, $\lambda_{0,0}$, is related to the polarity-polarizability of the indole environment (26), its bandwidth (BW, the width at two-thirds height) reports on the structural homogeneity of the site (27). For free Trp in homogeneous solutions, $\lambda_{0,0}$ ranges from 406 nm for a polar aqueous solution, to 411 nm for a completely non-polar hydrocarbon solvent (26). In proteins, $\lambda_{0,0}$ ranges from 403 to 420 nm (27). In micellar solutions, as for detergent-solubilized EII or IIC, the solvent can be either aqueous or non-polar, depending on whether a particular region of the protein surface is solvated by water or by the lipid tails of the surfactant. Hence, only $\lambda_{0,0}$ values outside the range 406–411 nm imply effective burial of the indole within the folds of EII or IIC.

- The highest spectral resolution is obtained with Trp residues buried in proteins having a unique conformation around the Trp site (e.g. Trp-72 of transhydrogenase from *Rhodospirillum rubrum*; BW = 3.2 nm) (28). Spectral broadening occurs on exposure of the aromatic ring to the solvent (BW = 5.7 nm for Trp in PG/water) and can be large (up to 15 nm), when the protein structure is not uniform at the Trp site, either because of local disorder or due to the presence of distinct conformers.

**Examples of phosphorescence spectra at 140 K, showing the range of spectral resolutions, are given in Fig. 2A for mutants Trp-97 (well resolved) and Trp-147 (broad). The values of $\lambda_{0,0}$ and BW, derived from the phosphorescence spectra at 140 K, are reported for all mutants in TABLE TWO. According to the $\lambda_{0,0}$ values, only residue 97 is located in a polar site (406.7 nm). For the other mutants, $\lambda_{0,0}$ is between 409.0 and 411.7 nm, wavelengths compatible with predominantly non-polar environments. These findings indicate that in none of the mutant proteins the chromophore is exposed to the aqueous phase, either because the $\lambda_{0,0}$ is at a higher wavelength compared to Trp free in solution ($\lambda_{0,0} > 406$ nm) or because in the case of Trp-97 it is much better resolved.**

![Phosphorescence Spectroscopy on EIImtl](Image)

TABLE ONE

<table>
<thead>
<tr>
<th>Mutant</th>
<th>$K_D^a$ (nM)</th>
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<tbody>
<tr>
<td>EIImtl-TL</td>
<td>70</td>
</tr>
<tr>
<td>Trp-66</td>
<td>141</td>
</tr>
<tr>
<td>Trp-97</td>
<td>1950</td>
</tr>
<tr>
<td>Trp-114</td>
<td>59</td>
</tr>
<tr>
<td>Trp-126</td>
<td>105</td>
</tr>
<tr>
<td>Trp-133</td>
<td>64</td>
</tr>
<tr>
<td>Trp-147</td>
<td>160</td>
</tr>
<tr>
<td>Trp-167</td>
<td>34</td>
</tr>
<tr>
<td>Trp-188</td>
<td>30</td>
</tr>
<tr>
<td>Trp-198</td>
<td>375</td>
</tr>
<tr>
<td>IICmtl-TL</td>
<td>358</td>
</tr>
<tr>
<td>Trp-97</td>
<td>2230</td>
</tr>
</tbody>
</table>

* Detergent-solubilized membrane vesicles; the errors in $K_D$, are typically below 10% (21).
Phosphorescence Spectroscopy on ElImtl

(BW < 5.7 nm). We also note that for Trp-97, phosphorescence and fluorescence spectra apparently lead to opposite conclusions regarding the polarity of the Trp microenvironment, polar for the former, non-polar for the latter. However, a blue-shifted fluorescence spectrum can also be indicative of a polar site that is too rigid to relax (shift to the red) during the fluorescence lifetime (26). The phosphorescence spectrum shows that for Trp-97 the latter interpretation is the correct one. A similar observation was made for Trp-72 in transhydrogenase from R. rubrum (28).

The BW of the 0,0-vibrational band is in most cases larger than for solvent-exposed Trp (BW = 5.7 nm). The lower spectral resolution in these mutants indicates multiple local configurations of the polypeptide and therefore structural heterogeneity. Exceptions are Trp-97, IIC-Trp-97 (BW = 3.4 nm), and Trp-114 (BW = 4.6 nm), which exhibit the best-resolved spectra. These sites are therefore rather homogeneous, implying an ordered local peptide structure. In the case of Trp-97, whose spectral resolution is among the highest ever reported for a globular protein fold, the spectrum is indicative of a unique, sharply defined structure around the chromophore, typical of tight β-sheet/barrel folds. Relative to Trp-97, the BW and local disorder increase progressively in the order Trp-114 < Trp-188 ~ Trp-66 ~ Trp-167 < Trp-198 ~ Trp-126 < Trp-133, to become large with Trp-147 (11.9 nm), suggesting a corresponding increase in conformational freedom at these sites.

Structural Flexibility from Thermal Spectral Relaxation in Buffer at 273 K—The gain in protein flexibility, as the temperature of glassy solutions is raised above the glass-transition state \( T_g (T_g < 200 \, K) \), allows the local structure to readjust itself around the triplet state dipole and achieve the lowest energy configuration. Thermal relaxation causes a red shift and broadening of the spectrum (10, 29). At near ambient temperature thermal population of near isoenergetic peptide conformations may also contribute to the loss in spectral resolution. As a rough guide, the larger the red shift and spectral broadening the more flexible and unstructured the protein site is. The average change in spectral energy (red shift) is generally indicated by the change in the center of gravity of the spectrum, \( \Delta \lambda_0 (T) \) \( \Delta \lambda_0 = (\Sigma \lambda_i P_i)/\Sigma P_i \), with \( P_i \) the phosphorescence intensity at \( \lambda_i \), a quantity that, unlike \( \lambda_{0,0} \), takes into account the conformational heterogeneity.

Examples of spectral relaxations occurring in changing from the glass state at 140 K to liquid buffer at 273 K are shown in Fig. 2 for mutants Trp-97 and Trp-147. In either case the spectrum becomes red-shifted and broad, relative to the glass state. However, the spectrum of Trp-97 maintains a clear vibronic structure even after thermal relaxation, indicating that the environment at position 97 is ordered and rigid also in

**TABLE TWO**

Phosphorescence spectral energies and bandwidth of the 0,0-vibrational peak of the single-tryptophan mutants

All values are in nanometers; typical errors are \( \lambda_{0,0} \pm 0.1 \, nm; \) BW, \( \pm 0.2 \, nm; \) \( \lambda_g \pm 0.3 \, nm.\)

<table>
<thead>
<tr>
<th>Mutant</th>
<th>( \lambda_{0,0}^a )</th>
<th>BW(^b)</th>
<th>( \Delta \lambda_0^T )</th>
<th>( \Delta \lambda_0^{mmt} )</th>
<th>( \Delta \lambda_0^{mmt} )</th>
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</thead>
<tbody>
<tr>
<td>ElImtl-Trp-66</td>
<td>411.7</td>
<td>6.8</td>
<td>442.0</td>
<td>447.1</td>
<td>+5.1</td>
</tr>
<tr>
<td>Trp-97</td>
<td>406.5</td>
<td>3.4</td>
<td>436.8</td>
<td>441.3</td>
<td>+4.5</td>
</tr>
<tr>
<td>Trp-114</td>
<td>409.0</td>
<td>4.6</td>
<td>439.3</td>
<td>445.3</td>
<td>+6.0</td>
</tr>
<tr>
<td>Trp-126</td>
<td>409.2</td>
<td>7.5</td>
<td>440.9</td>
<td>447.8</td>
<td>+6.4</td>
</tr>
<tr>
<td>Trp-133</td>
<td>409.9</td>
<td>8.5</td>
<td>440.1</td>
<td>446.6</td>
<td>+6.5</td>
</tr>
<tr>
<td>Trp-147</td>
<td>410.2</td>
<td>11.9</td>
<td>446.0</td>
<td>449.6</td>
<td>+8.3</td>
</tr>
<tr>
<td>Trp-167</td>
<td>409.0</td>
<td>6.8</td>
<td>439.3</td>
<td>448.4</td>
<td>+9.1</td>
</tr>
<tr>
<td>Trp-188</td>
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<td>440.3</td>
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</tr>
<tr>
<td>Trp-198</td>
<td>410.0</td>
<td>7.4</td>
<td>440.3</td>
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<tr>
<td>IICmtl-Trp-97</td>
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<td>3.4</td>
<td>436.8</td>
<td>441.3</td>
<td>+4.5</td>
</tr>
</tbody>
</table>

\(^a\) Values refer to the phosphorescence spectra in glasses \( (T = 140 \, K) \).
\(^b\) Center of gravity of the phosphorescence spectra, calculated according to \( \lambda_g = (\Sigma \lambda_i P_i)/\Sigma P_i \); \( P_i \) is the phosphorescence intensity at \( \lambda_i \). LT refers to measurements conducted at 140 K, HT refers to 273 K.
\(^c\) \( \Delta \lambda_0 (T) = \Delta \lambda_0^T - \lambda_g^T \).
\(^d\) Values refer to the shifts induced by the addition of 1 mM mannitol; \( \Delta \lambda_0^{mmt} = \lambda_g (273 K) - \lambda_g^{mmt} (273 K) \).
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Fluid solutions. On the contrary, upon thermal relaxation the spectrum of Trp-147 became considerably more red-shifted and broad; the loss of resolution reduced the 0,0-vibronic band into a mere shoulder. Thus, this region of ElI<sup>mtl</sup> is relatively flexible, free to sample a variety of local structures.

The magnitude of the spectral shift, Δλ<sub>5</sub>(T), for the different mutants is given in TABLE TWO. The parameter Δλ<sub>5</sub>(T) is not simply correlated to the local flexibility of the environment as, other things being equal, it depends on the blue or red nature of the low temperature (starting) spectrum. That is because the maximum red shift of a high energy site (blue low temperature spectrum) is greater than that of a low energy site. When the starting spectral energy is taken into account, by decreasing/increasing the shift depending on whether λ<sub>5</sub> (140 K) is to the blue/red of a reference state, we find that Δλ<sub>5</sub> (corrected) increases in the order Trp-97 < Trp-114 < Trp-126 < Trp-133 < Trp-198 < Trp-188 < Trp-167 < Trp-147, which should reflect the ranking in local fluidity of the matrix around the indole group.

Structural Fluidity and Homogeneity As Derived from the Phosphorescence Lifetime, τ<sub>i</sub> in Buffer at 273 K—Another sensitive parameter of the local protein/solvent mobility is the intrinsic phosphorescence lifetime, which decreases from about 6 s in rigid matrices to (sub) milliseconds in fluid solutions (11). Time-resolved measurements provide also information on the structural homogeneity of the protein site, as stable states of the protein ensemble differing in local flexibility will exhibit distinct lifetimes resulting in multiexponential phosphorescence decays.

The phosphorescence decays of Trp-97 and Trp-167 in buffer at 273 K are shown in Fig. 3 as extreme examples of decay kinetics among the various mutants. The decay of Trp-97 was the slowest and most uniform of all mutants, with an average lifetime (τ<sub>av</sub>) of 576 ms. This is over 1000-fold longer than τ of Trp exposed to the solvent (the lifetime of N-acetyl-l-tryptophanamide in the same medium) and is characteristic of Trp residues buried in ordered, rigid cores of the polypeptide normally rich in β secondary structure. On the contrary, the phosphorescence emission of Trp-167 was very short-lived and heterogeneous, with most of the intensity decaying with sub-millisecond lifetimes. Because the detergent gave a strong background signal during the first 200 μs (Fig. 3, buffer), the lifetime of shorter-lived components could not be determined with accuracy. The results, however, do emphasize that Trp-167 is in a very fluid site, possibly through contact with the non-polar tails of the surfactant, and that its emission is effectively quenched by collision with reactive side chains and/or quenching impurities in the solvent.

The decay was heterogeneous with every mutant, showing that each protein site probed by Trp adopts multiple local conformations in the micellar medium. The lifetime components, τ<sub>i</sub> and corresponding amplitu-

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**TABLE THREE**

Lifetimes and amplitudes of the phosphorescence decay

<table>
<thead>
<tr>
<th>Mutant</th>
<th>τ&lt;sub&gt;1&lt;/sub&gt;</th>
<th>α&lt;sub&gt;1&lt;/sub&gt;</th>
<th>τ&lt;sub&gt;2&lt;/sub&gt;</th>
<th>α&lt;sub&gt;2&lt;/sub&gt;</th>
<th>τ&lt;sub&gt;3&lt;/sub&gt;</th>
<th>α&lt;sub&gt;3&lt;/sub&gt;</th>
<th>τ&lt;sub&gt;av&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>EI&lt;sup&gt;mtl&lt;/sup&gt;-Trp-66</td>
<td>&lt;0.2</td>
<td>74</td>
<td>0.33</td>
<td>22</td>
<td>8.4</td>
<td>4</td>
<td>0.41–0.56</td>
</tr>
<tr>
<td>+ mtl</td>
<td>&lt;0.2</td>
<td>85</td>
<td>0.37</td>
<td>13</td>
<td>5.2</td>
<td>2</td>
<td>0.15–0.32</td>
</tr>
<tr>
<td>Trp-97</td>
<td>1.49</td>
<td>42</td>
<td>8.1</td>
<td>56</td>
<td>64</td>
<td>2</td>
<td>6.4</td>
</tr>
<tr>
<td>+ mtl</td>
<td>20</td>
<td>1.05</td>
<td>41</td>
<td>2.6</td>
<td>39</td>
<td>1.40–1.44</td>
<td></td>
</tr>
<tr>
<td>Trp-114</td>
<td>0.37</td>
<td>58</td>
<td>2.38</td>
<td>42</td>
<td>1.21</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ mtl</td>
<td>1.79</td>
<td>35</td>
<td>10</td>
<td>44</td>
<td>52</td>
<td>21</td>
<td>15.9</td>
</tr>
<tr>
<td>Trp-126</td>
<td>0.47</td>
<td>21</td>
<td>4.96</td>
<td>19</td>
<td>1.05–1.16</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ mtl</td>
<td>1.78</td>
<td>80</td>
<td>8.7</td>
<td>17</td>
<td>52</td>
<td>3</td>
<td>4.67</td>
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<tr>
<td>Trp-133</td>
<td>0.81</td>
<td>19</td>
<td>7.15</td>
<td>81</td>
<td>5.89</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ mtl</td>
<td>1.97</td>
<td>80</td>
<td>10</td>
<td>16</td>
<td>56</td>
<td>4</td>
<td>5.53</td>
</tr>
<tr>
<td>Trp-167</td>
<td>0.2</td>
<td>82</td>
<td>0.30</td>
<td>16</td>
<td>2.93</td>
<td>2</td>
<td>0.11–0.28</td>
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<tr>
<td>+ mtl</td>
<td>0.2</td>
<td>90</td>
<td>0.36</td>
<td>8</td>
<td>3.14</td>
<td>2</td>
<td>0.09–0.26</td>
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<tr>
<td>Trp-188</td>
<td>0.2</td>
<td>75</td>
<td>1.1</td>
<td>18</td>
<td>5.75</td>
<td>6.8</td>
<td>0.59–0.74</td>
</tr>
<tr>
<td>+ mtl</td>
<td>0.2</td>
<td>75</td>
<td>0.64</td>
<td>20</td>
<td>8.5</td>
<td>5</td>
<td>0.58–0.73</td>
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<tr>
<td>Trp-198</td>
<td>0.2</td>
<td>10</td>
<td>1.52</td>
<td>64</td>
<td>6.6</td>
<td>26</td>
<td>2.7</td>
</tr>
<tr>
<td>+ mtl</td>
<td>0.2</td>
<td>40</td>
<td>1.67</td>
<td>44</td>
<td>8.5</td>
<td>16</td>
<td>2.1–2.2</td>
</tr>
<tr>
<td>IIC&lt;sup&gt;mtl&lt;/sup&gt;-Trp-97</td>
<td>130</td>
<td>12</td>
<td>630</td>
<td>88</td>
<td>570</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ mtl</td>
<td>1.79</td>
<td>40</td>
<td>9.8</td>
<td>53</td>
<td>14</td>
<td>7</td>
<td>6.2</td>
</tr>
</tbody>
</table>

* Lifetime decays, τ<sub>i</sub> and corresponding amplitudes, α<sub>i</sub>, from 2–3 exponential component fitting of the phosphorescence decay.

* Because of the strong background emission during the initial 200 μs, lifetimes shorter than this value could not be determined with accuracy. In these cases, the range in average lifetime, τ<sub>av</sub> = Σα<sub>i</sub>τ<sub>i</sub>, is given, considering τ<sub>i</sub> either equal to 0 or to 0.2 ms. Errors in τ<sub>av</sub> values between repeated measurements are ±10%.
Effect of Mannitol Binding on the Fluorescence and Phosphorescence Emission

Mannitol binding changes the fluorescence and phosphorescence characteristics of some mutants considerably (Trp-97 and IIC-Trp-97), but leaves practically unaltered that of others (Trp-188). The change in fluorescent emission intensities was for most mutants <5% (data not shown), together with shifts of the maxima of <2 nm. The exceptions are Trp-66 with an 11% decrease in intensity and for the Trp at position 97 (Trp-97 and IIC-Trp-97) with 46% decrease in intensity together with a considerable red shift of 4 nm. According to the phosphorescent nature of the environment, are probably in contact with the hydrophobic tails of the surfactant.

The effect of mannitol binding on the thermal relaxation of the spectrum and on the phosphorescence lifetime for mutants Trp-97 and Trp-133 is shown in Figs. 4–6. The differences in $\Delta \lambda_{0,0}^\text{mtl}$ and $\Delta \tau_{av}^\text{mtl}$ report on the influence of mannitol binding on the local flexibility of the various sites of the IIC-domain. For Trp-133 the spectrum in buffer becomes sharper and blue-shifted ($\Delta \lambda_{0,0}^\text{mtl} = -0.8$ nm). This implies that the surrounding polypeptide structure becomes more ordered, in full accord with a better-resolved low temperature spectrum. The phosphorescence decay of Trp-133 becomes more uniform, and the average lifetime increased by ~6-fold (Fig. 6 and TABLE THREE), confirming

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an increased structural uniformity and rigidity when mannitol is bound. For Trp-97, binding of mannitol enhanced thermal relaxation of the spectrum ($\Delta \lambda_0^{\text{mtl}} = +2.4$ nm), which became broad and little resolved. The increase in flexibility was even more evident from the drastic almost 100-fold shortening of the phosphorescence lifetime, $\tau_{\text{av}}$, decreasing from 576 to 6.4 ms. According to the lifetime, the tight rigid core enveloping Trp-97 is lost in the mannitol-bound complex, suggesting that the process involves extensive unfolding of the local secondary structure. The spectral alteration induced by mannitol binding permitted us to attribute the shorting of the lifetime to a drastic increase in flexibility, rather than to potential intramolecular quenching reactions by cysteine, histidine, or tyrosine, triggered by the conformational change.

The results obtained with other mutants are summarized in TABLES TWO and THREE. For visual inspection, the changes induced by binding of mannitol on $\lambda_0$ and on $\tau_{\text{av}}$ are also displayed in Fig. 7. The two flexibility parameters are well correlated and report roughly the same trend on the structural influence of mannitol in various sites of the IIC-domain. Thus, a structuring effect is reported in the region of Trp-66, Trp-133, and Trp-147 by $\Delta \lambda_0^{\text{mtl}}$, in the region of Trp-126, Trp-133, and Trp-147 by $\Delta \tau_{\text{av}}^{\text{mtl}}$. The structure became looser in the region of Trp-66, Trp-97, Trp-114, Trp-167, and Trp-198 on the basis of both $\Delta \lambda_0^{\text{mtl}}$ and $\Delta \tau_{\text{av}}^{\text{mtl}}$. No change in the spectrum or lifetime was observed for Trp-188. Earlier, it was observed that the microenvironments of Trp-30 and (to a lesser extent) of Trp-42 changed upon mannitol binding (14). Taken their spectral characteristics into account these residues are therefore probably in contact with the hydrophobic core of the lipid bilayer.

In Fig. 7 the changes in microviscosity of the different residue positions induced by binding of mannitol are summarized. Until this study, changes in flexibility were expressed as the ratio of lifetimes with and without bound ligand ($\tau_{\text{av}}^{\text{mtl}}/\tau_{\text{av}}^0$, Fig. 1). We showed previously that phosphorescence spectroscopy is suitable for studying membrane proteins, provided that the protein samples are pure, oxygen removal is efficient, and the detergent does not introduce quenching components and has a low background luminescence (14). Our data show that the microenvironments of the studied Trp positions vary from exposed and flexible sites to a very rigid protein matrix and that mannitol binding induces large conformational changes in the IIC$^{\text{mtl}}$-domain at several of these positions.

For all mutants, except Trp-97 and IIC-Trp-97, the Trps are in non-polar environments, shielded from the aqueous phase. The non-polar nature of Trp-66, Trp-167, and Trp-188, and their high flexibility could be indicative for exposed positions in contact with the hydrophobic tails of the detergent belt that surrounds the IIC$^{\text{mtl}}$-domain. Residues 66 and 167 are predicted in helices II and IV, respectively (Fig. 1). Taking their spectral characteristics into account these residues are therefore probably in contact with the hydrophobic core of the lipid bilayer.

In Fig. 7 the changes in microviscosity of the different residue positions induced by binding of mannitol are summarized. Until this study, changes in flexibility were expressed as the ratio of lifetimes with and without bound ligand ($\tau_{\text{av}}^{\text{mtl}}/\tau_{\text{av}}^0$). Implementation of a sensitive charge-coupled device camera in the experimental setup made it possible to estimate the changes in emission spectra in the glass and fluid state, upon mannitol binding ($\Delta \lambda_0^{\text{mtl}}$), in a routine fashion. An advantage of this parameter, compared with $\tau_{\text{av}}^{\text{mtl}}/\tau_{\text{av}}^0$ is that the latter can be biased by nearby quenching groups, challenging the relation between $\tau_{\text{av}}$ and local flexibility. Interestingly, our data show that in the case of mannitol binding both the $\tau_{\text{av}}^{\text{mtl}}/\tau_{\text{av}}^0$ and $\Delta \lambda_0^{\text{mtl}}$ parameters correlate very well (Fig. 7).

**DISCUSSION**

Experimental knowledge about the dynamics of membrane-bound transport proteins during their catalytic cycle is scarce and limits the elucidation of the transport mechanism, including transporters of which the three-dimensional structure has recently been solved. The high sensitivity of tryptophan phosphorescence spectroscopy makes this an excellent tool to investigate membrane protein dynamics and heterogeneity.

In this investigation, we have characterized ten single-tryptophan mutants of the mannitol transporter (Elf$^{\text{mtl}}$) from E. coli, both in a mannitol-bound and unbound state. The Trp positions were chosen to probe various structural elements of the membrane-embedded IIC$^{\text{mtl}}$-domain (Fig. 1). We showed previously that phosphorescence spectroscopy is suitable for studying membrane proteins, provided that the protein samples are pure, oxygen removal is efficient, and the detergent does not introduce quenching components and has a low background luminescence (14). Our data show that the microenvironments of the studied Trp positions vary from exposed and flexible sites to a very rigid protein matrix and that mannitol binding induces large conformational changes in the IIC$^{\text{mtl}}$-domain at several of these positions.

FIGURE 7. Effects of mannitol binding on the flexibility of the IIC$^{\text{mtl}}$-domain at various positions. The changes induced by binding of mannitol are expressed as the ratio of the average lifetime with and without mannitol ($\tau_{\text{av}}^{\text{mtl}}/\tau_{\text{av}}^0$) and as the difference in the center of gravity, $\Delta \lambda_0^{\text{mtl}}$, of the phosphorescence spectra of the mutants with and without mannitol at 273 K. Error bars indicate the range of the ratios of the lifetime values, reported in TABLE THREE.
During the catalytic cycle of EII\textsubscript{mtl}, an interaction is established between the IIB\textsubscript{Amtl}- and IIC\textsubscript{mtl}-domains (1). A calorimetry study showed that, upon mannitol binding 50–60 residues become shielded from the aqueous phase (30). Because the effect was not observed for the IIC\textsubscript{mtl} mutant (lacking IIB\textsubscript{mtl}), the data were interpreted as a docking of the IIB\textsubscript{mtl}-domain onto the IIC\textsubscript{mtl}-domain. Except for Trp-97, the involvement of the IIB\textsubscript{mtl}-domains on the phosphorescent properties of EII\textsubscript{mtl} upon mannitol binding have not been investigated. The similarity of the phosphorescence data between Trp-97 and IIC-Trp-97, however, shows that large conformational changes occur in the IIC\textsubscript{mtl}-domain in the absence of the IIB\textsubscript{mtl}-domains. Binding of mannitol results in loss of structure in mutants Trp-66, Trp-97, Trp-114, Trp-167, and Trp-198, and the microenvironment of the Trp becomes more structured in Trp-126, Trp-133, and Trp-147. A chemical cross-linking study showed that a cysteine at position 124 can form a disulfide bridge with Cys-384 in the IIB\textsubscript{mtl}-domain (31). Possibly, the structuring observed for Trp-97 strongly suggest that it is embedded in a protein core around residue position 97. The large impact of the Phe-97 to Trp mutation on the ml binding affinity suggests that it is located close to the mannitol binding site. The localization of the studied tryptophan residues with respect to the mannitol binding site is currently investigated via fluorescence resonance energy transfer experiments using a chromophoric analogue of mannitol (15).

REFERENCES