

Membrane Protein–Ligand Interactions in *Escherichia coli* Vesicles and Living Cells Monitored via a Biosynthetically Incorporated Tryptophan Analogue[†]

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ABSTRACT: This paper presents a deceptively straightforward experimental approach to monitoring membrane protein–ligand interactions in vesicles and in living *Escherichia coli* cells. This is achieved via the biosynthetic incorporation of 7-azatryptophan, a tryptophan analogue with a red-shifted absorption spectrum, allowing collection of the emission signal of the target protein in a high tryptophan background via red-edge excitation. The approach is demonstrated for the mannitol permease of *E. coli* (EII^{mtl}), an integral membrane protein of 637 amino acids, including four tryptophans, and single-tryptophan mutants of EII^{mtl}. By using a tryptophan auxotroph, a high level of 7-azatryptophan incorporation in EII^{mtl} was achieved. The change in emission signal of the purified enzyme upon mannitol binding (–28%) was 4-fold larger than with EII^{mtl} containing tryptophan, demonstrating the known higher sensitivity of this analogue for changes in the microenvironment [Schlesinger, R. (1968) *J. Biol. Chem.* 243, 3877–3883]. Changes in emission signal could also be monitored (–5%) when the enzyme was situated in vesicles, although it constituted only 10–15% of the total cytoplasmic membrane fraction. Of the five single-tryptophan mutants, the emission signal of the mutant with 7-azatryptophan at position 198 was the most sensitive for mannitol binding. Changes in emission signal not only were observed in vesicles (–18%) but also could be monitored in viable cells (–5%). The fact that only modest expression levels and no protein purification are needed makes this a useful approach for the characterization of numerous protein systems under in vitro and in vivo conditions.

Tryptophan fluorescence spectroscopy is a potentially powerful approach to investigating membrane proteins (1–4). The easy introduction of this probe at the DNA level and the sensitivity of tryptophan (Trp)¹ to changes in microenvironment facilitate the generation of structural and dynamic information at high resolution even at positions which are not solvent-exposed and, therefore, cannot be studied by the cysteine labeling approach (5, 6). Widespread use of Trp fluorescence spectroscopy in membrane protein research has been hampered however by the difficulty of eliminating high background fluorescence, a prerequisite for quenching and especially for time-resolved lifetime and anisotropy measurements. Recently, we have addressed this issue and developed procedures for avoiding high background fluorescence (4, 7, 8).

Membrane vesicles are an ideal system for investigating membrane protein–ligand interactions, yet the cysteine labeling and the Trp spectroscopy methodologies usually necessitate the solubilization and purification of the labeled protein, followed eventually by its reconstitution back in proteoliposomes. Besides being laborious, the purification often affects the functional and oligomeric state of the membrane protein and its interactions with other proteins in the case of multiprotein complexes. Moreover, the difficulty of unidirectional reconstitution in proteoliposomes hampers the specific targeting of ligands to one of the two sides of the protein in such systems as pores, transporters, or proteins involved in signal transduction.

To overcome these disadvantages, we have explored the biosynthetic incorporation of 7-azatryptophan (7-ATrp) with a red-shifted absorption spectrum in a membrane protein. Targeted incorporation was achieved by replacing Trp with the Trp analogue prior to induction and overexpression of the membrane protein (9). The specific excitation of 7-ATrp at 310 nm allowed us to monitor changes in the emission signal of the 7-ATrp-containing membrane protein in vesicles and even in whole cells. The approach will be demonstrated

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¹ Abbreviations: EII^{mtl}, mannitol-specific transporting and phosphorylating enzyme from *E. coli*; ISO, inside-out; 7-ATrp, 7-azatryptophan; DOC, sodium deoxycholate; decyl-PEG, decylpoly(ethylene glycol) 300; PEP, phosphoenolpyruvate.

with the mannitol permease (EII^{mtl})² of *Escherichia coli* containing four tryptophans, and five single-Trp mutants of this enzyme.

MATERIALS AND METHODS

Chemicals and Reagents. DL-7-ATrp and DOC were purchased from Sigma. Imidazole (spectroscopic grade) was from Fluka. Ni-NTA resin was from Qiagen Inc. DOC was purified as described previously (7). Decyl-PEG was purified as described for C₁₀E₅ (7).

Plasmids and Bacterial Strains. Plasmids of the single Trp mutants of EII^{mtl} (W30, W42, W109, and W117) containing the λ -P_R promoter and the cI₈₅₇ temperature sensitive repressor were used (4). The W198 mutant was created, starting from the Trp-minus plasmid in a similar way by using the 5'-CTG AAT TCC CAG CGG CGA CCA GAT ACC-3' primer. This primer also introduced an additional EcoRI restriction site. Construction of the plasmid of the N-terminal His-tagged EII^{mtl}, also containing the λ -P_R promoter and the cI₈₅₇ temperature sensitive repressor, will be published elsewhere. The *E. coli* Trp auxotroph M5219 (λ cI₈₅₇ lysogen, *Trp_{am}*) (10), obtained from BCCM/LMBP (Gent, Belgium), was used for the incorporation of 7-ATrp in EII^{mtl}. This strain is not a deletion mutant for EII^{mtl}. However, as long as the cells are not grown on mannitol, the chromosomal expression level will be very low in comparison with the level of temperature-induced expression from the plasmid.

Growth and Induction of Bacteria. A 20 mL overnight culture of M5219 in LB medium was added to 1 L of M9 minimal medium (11) supplemented with 0.5% glucose, 0.1% thiamine, 0.1 mM CaCl₂, 1 mM MgCl₂, 0.01 mM FeSO₄, trace elements (12), 100 μ g/mL ampicillin, and 1 mM L-Trp. Growth by shaking at 30 °C was continued until the OD₆₀₀ reached 0.6–0.7, followed by collection of the cells by centrifugation and resuspension in the same volume of medium, without L-Trp. Shaking at 30 °C was continued for 30 min, followed by the introduction of 2 mM DL-7-ATrp, dissolved in dilute NaOH. After 15 min at 30 °C, the temperature was raised to 42 °C and incubation was continued for 2.5 h. Cells were collected by centrifugation, and washed with 25 mM Tris-HCl buffer (pH 7.5).

ISO Vesicle Preparation (13). A cell suspension in cold 20 mM Tris-HCl (pH 7.5), 1 mM DTT, 1 mM NaN₃, 1 mM MgCl₂, 1 mM PMSF, approximately 0.2 mg/mL RNase, and DNase (5 mL of buffer per gram of wet cells) was passed once through a French press (Carver Inc.) at 10 000 psi. After addition of 2 mM EDTA, the suspension was centrifuged for 10 min at 23000g and 4 °C. The supernatant was carefully separated from the pellet and centrifuged for 90 min at 170000g. The pellet was homogenized by using a potter tube in 20 mM Tris-HCl (pH 8.4), 1 mM DTT, and 1 mM NaN₃. After ultracentrifugation, the pellet was again homogenized

in the same buffer with a volume (milliliters) equal to the weight of the wet cells (grams). These vesicle stock solutions were stored in liquid nitrogen or at –80 °C in small quantities. The percentage of EII^{mtl} of the total cytoplasmic protein fraction was determined by scanning [Image Master (Pharmacia Biotech, Uppsala, Sweden)] Coomassie Blue-stained SDS–PAGE gels loaded with different amounts of vesicles.

EII^{mtl}-His Purification. Since fluorescent impurities present in detergents absorb also strongly at 310 nm (7), purified detergents were used during the isolation of 7-ATrp containing EII^{mtl}-His. A DOC extract (14) of EII^{mtl}-His in 20 mM Tris-HCl (pH 8.4), 0.5% DOC, 300 mM NaCl, 2 mM reduced glutathione, and 10 mM imidazole was mixed with Ni-NTA resin (1.5 mL/mL of vesicle solution). After 1 h at 4 °C, the suspension was poured into a column. The column was washed with 4 column volumes of this DOC buffer, and washing was continued after replacing DOC with 0.35% (v/v) spectroscopically pure decyl-PEG detergent. After addition of 6 column volumes, no fluorescence was washed from the column and the protein was eluted batchwise in this buffer by 150 mM imidazole. The purification procedure was performed in the cold room. SDS–PAGE showed that the enzyme was >95% pure.

EII^{mtl} Activity and Concentration Measurements. The PEP-dependent mannitol phosphorylation activity of EII^{mtl} was determined as described previously (4). For 7-ATrp-containing wild-type and W30 EII^{mtl}, turnover numbers of 4000 and 2250 min⁻¹ were found, comparable with the activity observed for the Trp-containing enzymes, 4100 and 2050 min⁻¹, respectively (4).

EII^{mtl} concentrations in the vesicle stock solutions were determined by the flow-dialysis technique as described previously (15). For the EII^{mtl}-His, W30 and W198 vesicle enzyme concentrations of 20, 27, and 23 μ M and K_D values for the EII^{mtl}–mannitol complex of 110, 50, and 300 nM were found, respectively.

Fluorescence Measurements. Fluorescence spectra were recorded at room temperature on a SLM-Aminco SPF-500 fluorometer adjusted to a 2 nm excitation band-pass and a 5 nm emission band-pass. The buffer that was used consisted of 20 mM Tris-HCl (pH 7.5) and 2 mM reduced glutathione, and the enzyme concentrations were 250–400 nM. All emission spectra were corrected for instrument response and for buffer. Changes in emission spectra induced by mannitol were calculated after integration of the spectra from 320 to 500 nm. The emission signals of the vesicles and whole cells were stable during the experiments and were not influenced by photobleaching or sedimentation. In addition, the results could be reproduced by using a different batch of vesicles or cells.

Stopped-Flow Fluorescence Spectroscopy. An Applied Photophysics SX-17MV stopped-flow spectrofluorimeter was used operating at 25.0 °C. Excitation was at 305 nm, and the emitted light was passed through a WG345 Schott cutoff filter (cutoff at 345 nm). The vesicle stock solution was diluted 100-fold in 20 mM Tris-HCl (pH 8.4), 1 mM NaN₃, and 2 mM reduced glutathione and mixed with an equal volume of mannitol in the same buffer. The final enzyme concentration was 100–150 nM, depending on the batch of vesicles that was used. Up to 10 spectra were averaged and analyzed by the nonlinear regression analysis software

² Nomenclature of the enzymes: EII^{mtl}, wild-type enzyme containing tryptophans at positions 30, 42, 109, and 117; W30, W42, W109, and W117, single-Trp EII^{mtl} mutants with Trp present at positions 30, 42, 109, and 117, respectively, with the other three tryptophans having been replaced with phenylalanine; W198, EII^{mtl} mutant in which all four tryptophans at positions 30, 42, 109, and 117 have been replaced with phenylalanines and the phenylalanine at position 198 has been replaced with Trp; EII^{mtl}-His, wild-type EII^{mtl} extended by an N-terminal tail of six histidine residues.

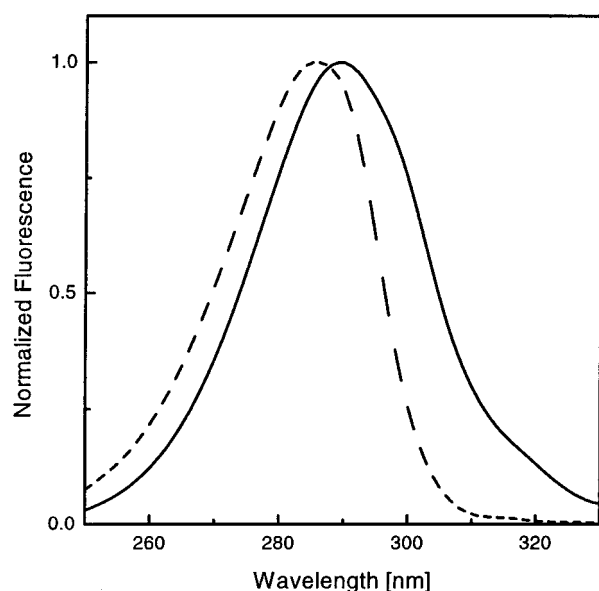


FIGURE 1: Excitation spectra of purified EII^{mtl}. For 7-ATrp-containing EII^{mtl}-His (solid line), emission was at 380 nm, and for tryptophan-containing EII^{mtl} (4) (dashed line), emission was at 350 nm. Excitation and emission band-passes were at 4 nm. Spectra were normalized to their maxima.

supplied with the stopped-flow machine. Since the reactions were performed under pseudo-first-order conditions, the association rate constant was calculated by plotting the reciprocal relaxation time (k_{obs}) versus the sugar concentration (16).

RESULTS

The *E. coli* Trp auxotroph M5219 was grown at 30 °C in M9 minimal medium supplemented with 1 mM L-Trp to an OD₆₀₀ of 0.7. Cells were collected, washed, and resuspended in M9 medium without L-Trp, and shaking was continued at 30 °C for 30 min to remove residual L-Trp. DL-7-ATrp (2 mM) was introduced, and after 15 min, the induction was initiated by raising the temperature to 42 °C and shaking continued for 2.5 h. The higher temperature resulted in almost no further cell growth since the OD₆₀₀ increased only 20%. ISO vesicles were isolated by passing the cells through a French press, and EII^{mtl}-His was purified to homogeneity via Ni-NTA agarose chromatography. A shoulder at >300 nm is clearly visible in the excitation spectrum of the purified enzyme, representing 7-ATrp (Figure 1). Attempts to determine the percentage of 7-ATrp incorporation by amino acid analysis were not successful due to the low content of Trp (<1% of the amino acids is Trp) and coelution of other hydrolysis products with the same retention time as 7-ATrp. The limited solubility of EII^{mtl} (micromolar concentrations) and 11 tyrosine residues in EII^{mtl}, including a tyrosinate, prevented an estimation of the efficiency of analogue incorporation via UV spectroscopy (17). However, recording excitation spectra at different emission wavelengths provided this information. Changing the emission wavelength from 380 nm (Figure 1) to 350 nm, where Trp exhibits a significant signal (4), resulted in a blue shift of the excitation spectrum between 300 and 320 nm of less than 1 nm, indicative of a high level of 7-ATrp incorporation.

The emission spectrum of this sample, excited at 310 nm, and the effect of mannitol are shown in Figure 2A. The

emission signal of the 7-ATrp-containing EII^{mtl} decreased 28% upon binding mannitol. Interestingly, the binding of mannitol can also be monitored without enzyme purification by directly using ISO vesicles (Figure 2B). The emission signal decreased 5% upon the introduction of mannitol. The 6-fold lower change in emission signal in ISO vesicles compared to that in the purified sample is due to a background signal resulting from scattering of the vesicles, autofluorescence caused by chromophores absorbing at 310 nm like NADH, and of proteins coexpressed during the EII^{mtl} induction at 42 °C. We showed earlier using four purified single-Trp mutants of EII^{mtl} that the Trp at position 30 (W30 mutant) is responsible for the change in emission found in wild-type EII^{mtl} upon mannitol binding (4). A similar trend was observed for these mutants expressed in the presence of 7-ATrp. Only the spectrum of W30 ISO vesicles changed 7% upon the introduction of mannitol (Figure 2C).

Both the periplasmic and the cytoplasmic face of EII^{mtl} exhibit high affinity for mannitol and the inhibitor perseitol (18). The results with ISO vesicles presented in Figure 2 enable us to study specifically the transient kinetics of the sugar binding process at the cytoplasmic side by using stopped-flow fluorescence spectroscopy. Mixing W30 ISO vesicles with excess (1–10 μM) mannitol resulted in a monoexponential decrease of the emission signal. The final enzyme concentration was only 135 nM. Plotting of the reciprocal relaxation time k_{obs} versus the mannitol concentration resulted in a linear relationship, giving an association rate constant of $(6.9 \pm 0.3) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (Figure 3). Similar experiments performed with perseitol, an inhibitor which is specifically bound by EII^{mtl} (18), yielded an association rate constant of $(2.7 \pm 0.2) \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ (Figure 3). When these vesicles were mixed with sorbitol, a sugar not interacting with EII^{mtl}, no change in fluorescence signal was observed.

Besides the four single-Trp EII^{mtl} mutants with one Trp at a wild-type position, the single-Trp mutant W198, with the four tryptophans replaced with phenylalanine and a new Trp introduced at position 198, was also investigated. W198, expressed in M5219, exhibited a lower affinity for mannitol ($K_D = 300 \text{ nM}$) than wild-type EII^{mtl} or the single-Trp mutants discussed above, while the PEP-dependent mannitol phosphorylation activity was comparable. The addition of mannitol to ISO vesicles of 7-ATrp-containing W198 had a large effect on the emission signal (–18%, Figure 2D). The K_D for mannitol was determined by directly titrating these vesicles with mannitol and monitoring the changes in emission signal at 366 nm, yielding a K_D of $240 \pm 20 \text{ nM}$ (Figure 4) in good agreement with the results from the flow-dialysis binding assay.

Given the large fluorescence change in emission of W198 ISO vesicles upon binding of mannitol, we have explored whether this interaction can also be monitored by using viable cells. Figure 2E shows that a 5% decrease in the emission signal is observed when mannitol is added to these cells. A similar result was found for perseitol (–5%), but no changes in the signal were observed if the experiment was repeated with the same concentration of glucose or sorbitol, sugars not interacting with EII^{mtl}. The kinetics of mannitol binding by whole cells are complex since the sugar has to pass the outer membrane and the phosphorylation state of the enzyme cannot be controlled. Also, rebinding of mannitol at the

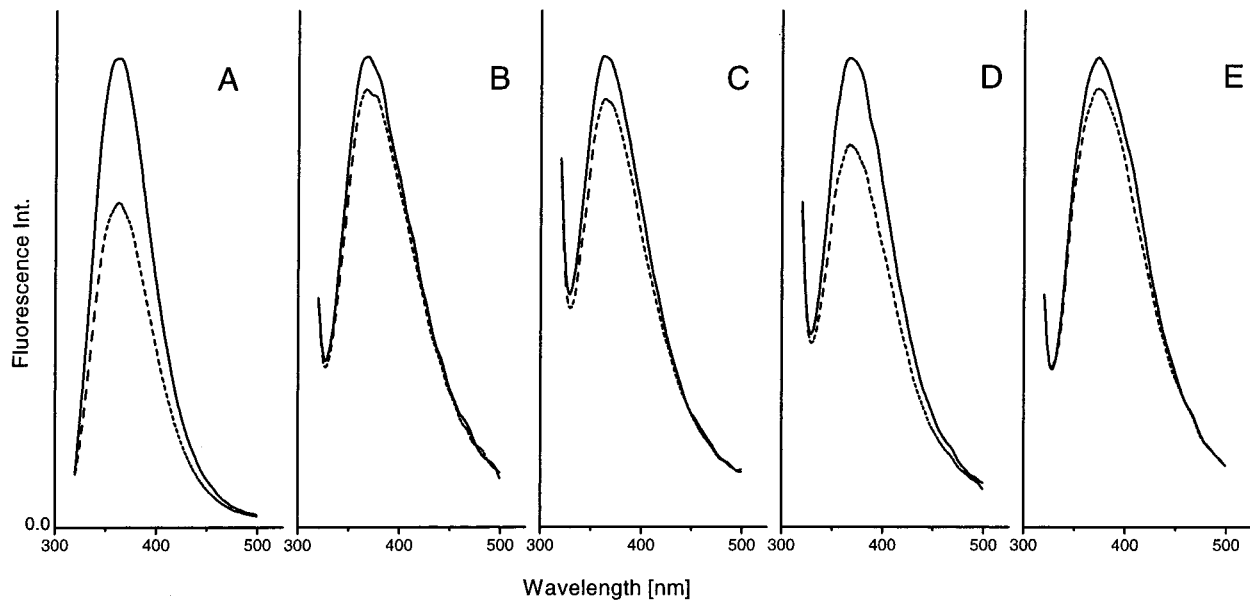


FIGURE 2: Emission spectra of 7-A-Trp-containing EII^{ml} samples and the effect of mannitol. (A) Purified EII^{ml}-His without (solid line) and with 50 μ M mannitol (dashed line). (B) EII^{ml}-His ISO vesicles without (solid line) and with 60 μ M mannitol (dashed line). (C) W30 ISO vesicles without (solid line) and with 60 μ M mannitol (dashed line). (D) W198 ISO vesicles without (solid line) and with 60 μ M mannitol (dashed line). (E) W198-containing EII^{ml} whole *E. coli* cells without (solid line) and with 100 μ M mannitol (dashed line).

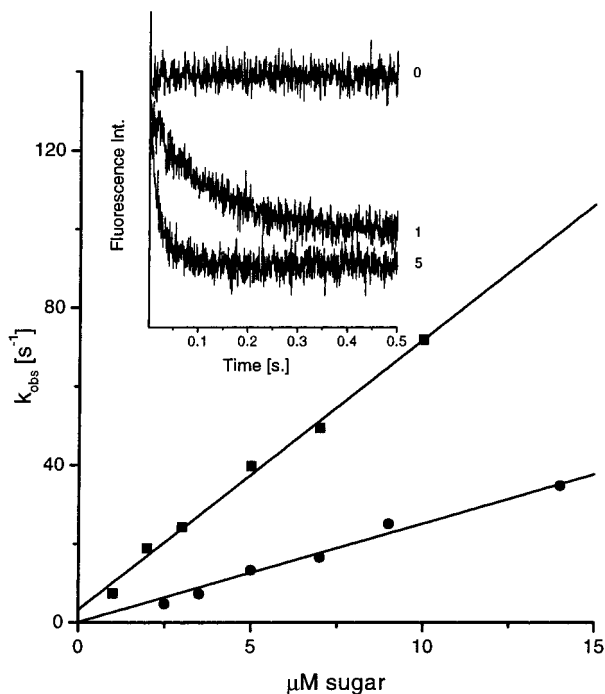


FIGURE 3: Relationship between k_{obs} and mannitol (■) and perseitol (●) concentrations using W30 ISO vesicles. The inset shows transient kinetic traces of W30 ISO vesicles (135 nM enzyme) mixed with 0, 1, and 5 μ M mannitol (final concentrations). The traces are the average of results from five reactions.

cytoplasmic side is possible since only 50% of the transported sugar becomes phosphorylated (19). Nevertheless, the experiments demonstrate that this protein–ligand interaction can be monitored *in vivo*.

DISCUSSION

A number of Trp analogues, including 2-azatryptophan, 7-azatryptophan, 5-hydroxytryptophan, and 4- and 5-fluorotryptophan, can be biosynthetically incorporated, as demonstrated in several reports in the past forty years (9, 20–

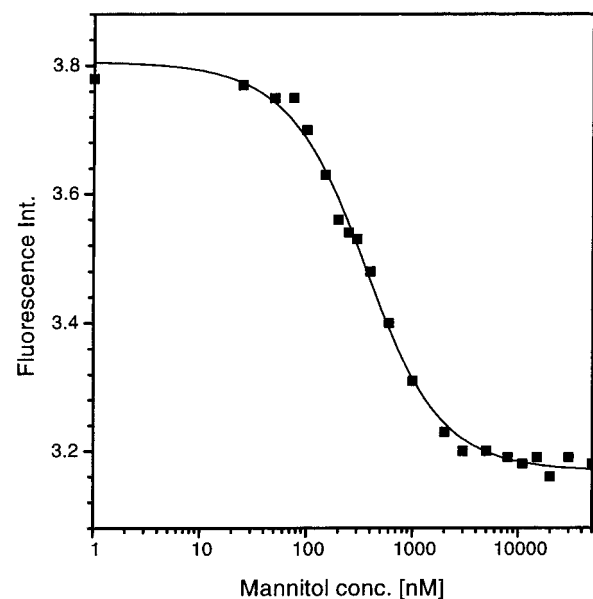


FIGURE 4: Titration of W198 ISO vesicles with mannitol. The titration (250 nM enzyme) was performed at 25.0 $^{\circ}$ C by stepwise increases of the mannitol concentration and monitoring the emission signal at 366 nm. Excitation was at 310 nm.

25). Efficient incorporation of Trp analogues in a specific protein can be achieved when its expression is under the control of an inducible promoter as demonstrated in 1992 by Szabo and Ross (17, 26). In this approach, an *E. coli* Trp auxotroph is grown in a Trp-containing medium. After Trp is replaced in the medium for the analogue, protein expression is induced. Trp analogues such as 2-azatryptophan, 7-azatryptophan, and 5-hydroxytryptophan exhibit red-shifted absorbance spectra, enabling specific red-edge excitation of these analogues in a high Trp background (9, 22). This approach has been used successfully for investigating protein–protein interactions where one of the proteins contains the Trp analogue, and in protein–DNA interactions where the high level of absorption of DNA in UV, acting as

an internal filter, complicates the reliable monitoring of Trp emission (9). Also, the effect of the subtle change in Trp side chain structure on protein stability and activity has been investigated (27–29). In all of the above examples, purified water-soluble proteins have been used.

We demonstrate in this paper that 7-ATrp can be efficiently incorporated in the wild type and single-Trp mutants of EII^{mtl}, an integral membrane protein, by using the M5219 Trp auxotroph. The incorporation of 7-ATrp did not affect the mannitol phosphorylation activity of EII^{mtl} or its affinity for mannitol. We showed earlier that binding of mannitol to purified wild-type EII^{mtl} containing Trp results in an 8% increase in the emission signal (4). The emission signal of the purified 7-ATrp-containing EII^{mtl} decreased 28% (Figure 1A). This clearly demonstrates the much higher sensitivity of the emission signal of 7-ATrp compared to that of Trp for changes in the microenvironment (21). The mechanism resulting in an increase in Trp fluorescence versus a decrease in 7-ATrp fluorescence upon mannitol binding is currently being investigated.³ Although the 7-ATrp EII^{mtl} accounts for only 10–15% of the total bacterial membrane protein fraction, the change in 7-ATrp emission induced by mannitol binding can be clearly determined in ISO vesicles upon excitation at 310 nm (Figure 2B–D). Most likely, this relative change in emission signal can be improved somewhat further if an expression system is used that is based on an *E. coli* chromosome-directed protein synthesis induction system, stopping cell growth upon induction, and preventing incorporation of 7-ATrp in other *E. coli* proteins. For this reason, we are currently exploring the use of the T7 promoter system (30).

Direct monitoring of protein–ligand interactions in vesicles by using Trp fluorescence has been reported before for GalP, an *E. coli* protein which is overexpressed to extremely high levels of up to 60% of the total cytoplasmic membrane protein fraction (31). The current results indicate that the combination of the red-edge excitation of the 7-ATrp-containing target protein in vesicles and the high sensitivity of 7-ATrp for changes in the microenvironment (21) allow in situ spectroscopy and stopped-flow kinetics at moderate protein expression levels. Since protein purification steps using detergent are not needed, this approach can be particularly important for proteins that are functional in a higher oligomeric state or a multiprotein complex, held together by weak interactions. In some such systems, purification can result in dissociation and/or loss of essential subunits.

Of the various EII^{mtl} mutants that were investigated, the W198 mutant exhibited the largest change in 7-ATrp emission intensity upon mannitol binding (Figure 2D). Residue 198 is located in a part of the protein known to be involved in mannitol binding and translocation (32, 33), and the large change in fluorescence induced by mannitol is in line with this. The change in emission signal could even be determined by red-edge excitation of *E. coli* cells expressing W198, demonstrating for the first time that a protein–ligand

interaction can be monitored in vivo by using intrinsic Trp fluorescence. The location and movement of proteins can be monitored in cells with confocal fluorescence spectroscopy. However, the spacial resolution of a confocal microscope is in the same range as the size of an *E. coli* cell (1–2 fL) (34). This prevents a more sensitive monitoring of the mannitol-induced emission change in *E. coli* cells containing W198 by focusing on the cytoplasmic membrane. If one uses larger eukaryotic cells such as yeast, for which a Trp auxotroph has been reported (35), biosynthetic incorporation of Trp analogues in combination with multiphoton excitation of 7-ATrp offers a potentially sensitive system for studying in vivo conformational changes of a specific protein by confocal microscopy.

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³ Studies in which ligand-induced changes in emission have been compared for Trp- and 7-ATrp-containing proteins are rare, and to our knowledge, the increase in the emission of Trp-containing protein versus a decrease in that of the 7-ATrp-containing protein has not been reported before.

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