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Isolation of *Escherichia coli* Mannitol Permease, EII^{mtl}, Trapped in Amphipol A8-35 and Fluorescein-Labeled A8-35

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Abstract Amphipols (APols) are short amphipathic polymers that keep integral membrane proteins water-soluble while stabilizing them as compared to detergent solutions. In the present work, we have carried out functional and structural studies of a membrane transporter that had not been characterized in APol-trapped form yet, namely EII^{mtl}, a dimeric mannitol permease from the inner membrane of *Escherichia coli*. A tryptophan-less and dozens of single-tryptophan (Trp) mutants of this transporter are available, making it possible to study the environment of specific locations in the protein. With few exceptions, the single-Trp mutants show a high mannitol-phosphorylation activity when in membranes, but, as variance with wild-type EII^{mtl}, some of them lose most of their activity upon solubilization by neutral (PEG- or maltoside-based) detergents. Here, we present a protocol to isolate these detergent-sensitive mutants in active form using APol A8-35. Trapping with A8-35 keeps EII^{mtl} soluble and functional in the absence of detergent. The specific phosphorylation activity of an APol-trapped Trp-less EII^{mtl} mutant was found to be $\sim 3\times$ higher than the activity of the same protein in dodecylmaltoside. The preparations are suitable both for functional and for fluorescence spectroscopy studies. A fluorescein-labeled version of A8-35 has been synthesized and characterized. Exploratory studies were conducted to examine the environment of specific Trp locations in the transmembrane domain of EII^{mtl} using Trp fluorescence quenching by water-soluble quenchers and by the fluorescein-labeled APol. This approach has the potential to provide information on the transmembrane topology of MPs.

Keywords Membrane protein · Fluorescent amphipol · Fluorescence quenching · Förster resonance energy transfer

Abbreviations

2D	Two dimensional
A8-35	Poly(sodium acrylate) based amphipol comprising 35 % of free carboxylate, 25 % of octyl chains, 40 % of isopropyl groups, and whose weight average molar mass is $\sim$4.3 kDa
A8-75	Poly(sodium acrylate) based amphipol comprising 75 % of free carboxylate, 25 % of octylchains, whose weight average molar mass is $\sim$4 kDa
APol	Amphipol
B_{tot}	Total amount of binding sites
$C_{10}E_5$	Decylpentaethylene glycol ether
C_{10}-PEG	Decylpoly(ethyleneglycol) 300
CBB	Coomassie brillant blue
DABCO	1,4-Diazabicyclo[2.2.2]octane
DCI	*N,N*-Dicyclohexylcarbodiimide
DMF	Dimethylformamide
DOC	Deoxycholate
DTT	Dithiothreitol
EII^{mtl}	Dimeric mannitol permease from the inner membrane of *Escherichia coli*

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FAPol	Fluorescently-labeled A8-35
$FAPol_{fluo}$	Fluorescein-labeled A8-35
FITC	Fluorescein isothiocyanate
FRET	Förster resonance energy transfer
IIA^{mtl}, IIB^{mtl}	Cytoplasmic A and B domains of EII^{mtl}, respectively
IIC^{mtl}	Transmembrane C domain of EII^{mtl}
ISO	Inside-out
K_D	Dissociation constant
MP	Membrane protein
NBD	7-Nitrobenz-2-oxa-1,3-diazol-4-yl
NTA	Nitrilotriacetic acid
PAA	Poly(acrylic acid)
SDS-PAGE	Sodium dodecylsulfate-polyacrylamide gel electrophoresis
TL	Trp-less EII^{mtl}, in which the four native Trp residues are replaced by Phe
TMHI	The first putative transmembrane helix of IIC^{mtl}
tOmpA	The transmembrane domain of outer membrane protein A from *E. coli*
Trp	Tryptophan
UAPol	A8-35 grafted with an amino arm
W36, W37, W38, W167, and W188	Single-Trp-containing EII^{mtl} mutants based on Trp-less EII^{mtl}
wt EII^{mtl}	Wild-type EII^{mtl}, with Trp residues at positions 30, 42, 109, and 117

Introduction

Integral membrane proteins (MPs) are usually kept soluble in aqueous solutions using detergents (le Maire et al. 2000). Detergents, however, tend to inactivate them (for reviews and discussions about the origin(s) of the effect, see e.g., Bowie 2001; Garavito and Ferguson-Miller 2001; Gohon and Popot 2003; Popot 2010; Rosenbusch 2001). Amphipathic polymers called amphipols (APols) have proven to be efficient substitutes to detergents (Popot 2010, 2011; Tribet et al. 1996; Zoonens and Popot 2014). By adsorbing specifically onto the transmembrane region of MPs (Althoff et al. 2011; Catoire et al. 2009; Etzkorn et al. 2014; Huynh et al. 2014; Liao et al. 2013, 2014; Perlmutter et al. 2014; Zoonens et al. 2005), they keep MPs water-soluble and, as a rule, strongly stabilize them as compared to their detergent-solubilized counterparts (reviewed in refs. Popot 2010; Popot et al. 2003, 2011; Zoonens and Popot 2014). The conformational transitions of some MPs, like bacteriorhodopsin (BR) (Dahmane et al. 2013; Gohon et al. 2008) and the nicotinic acetylcholine receptor (Martinez et al. 2002), are very similar in the membrane and after trapping with APols, and so are the pharmacological properties of various G protein-coupled receptors (GPCRs) (reviewed in Banères et al. 2011; Mary et al. 2014). The enzymatic activities of cytochrome bc_1 and of the photoreceptor-specific ABC transporter ABCA4 are essentially identical in detergent solution and after trapping with APols (Charvolin et al. 2014; Tsybovsky et al. 2013). On the contrary, the enzymatic cycle of the sarcoplasmic calcium ATPase is reversibly inhibited (Champeil et al. 2000; Picard et al. 2006). Because the transmembrane surfaces of BR, the nicotinic acetylcholine receptor and GPCRs undergo only limited rearrangements during the functional cycle, whereas that of the sarcoplasmic calcium ATPase experiences larger conformational changes, it has been speculated that APols may damp large-scale transmembrane movements, which may contribute to their stabilizing effect (Picard et al. 2006; Popot et al. 2003, 2011; Popot 2010). This hypothesis is consistent with the observation that APols stabilize *E. coli*'s outer membrane protein A (OmpA) by raising the free energy barrier that opposes unfolding (Pocanschi et al. 2013). It has received some further support from molecular dynamics calculations showing that the environment provided by APol A8-35 is more viscous than that of a detergent micelle or even a lipid bilayer (Perlmutter et al. 2011; Tehei et al. 2014), and that the breathing movements of *E. coli*'s outer MP OmpX are damped in A8-35 as compared to the detergent dihexanoylphosphocholine (Perlmutter et al. 2014). Given the broad range of applications APols are being put to (reviewed by Popot et al. 2011; Zoonens and Popot 2014), it is of great interest to better understand their interactions with MPs and how they may or may not affect the stability and functionality of various types of MPs. In this communication, we explore the ability of A8-35, a polyacrylate-based APol (Tribet et al. 1996), to substitute to detergents and to keep *E. coli*'s mannitol transporter, EII^{mtl}, and several of its detergent-sensitive mutants water-soluble and functional.

EII^{mtl} is an inner membrane protein. It belongs to the family of phosphoenolpyruvate-dependent phosphotransferase transporters, which is present only in prokaryotic organisms. EII^{mtl} transports mannitol from the periplasm to the cytoplasm, while phosphorylating it. Two other kinases are involved in this process, namely Enzyme I and HPr (Robillard and Broos 1999). EII^{mtl} is composed of three domains, the cytoplasmic A (IIA^{mtl}) and B (IIB^{mtl}) domains and the transmembrane C domain (IIC^{mtl}). The structures of the cytoplasmic domains have been solved by X-ray crystallography and NMR spectroscopy (Legler et al. 2004; van Montfort et al. 1998). Structural information about IIC^{mtl} is restricted to topology models (Seybold et al. 1969; Sugiyama et al. 1991; Vervoort et al. 2005) and a 5-Å 2D projection map (Koning et al. 1999). Wild-type (wt) EII^{mtl} comprises four tryptophan (Trp) residues. A Trp-less (TL) version of EII^{mtl} has been created, in

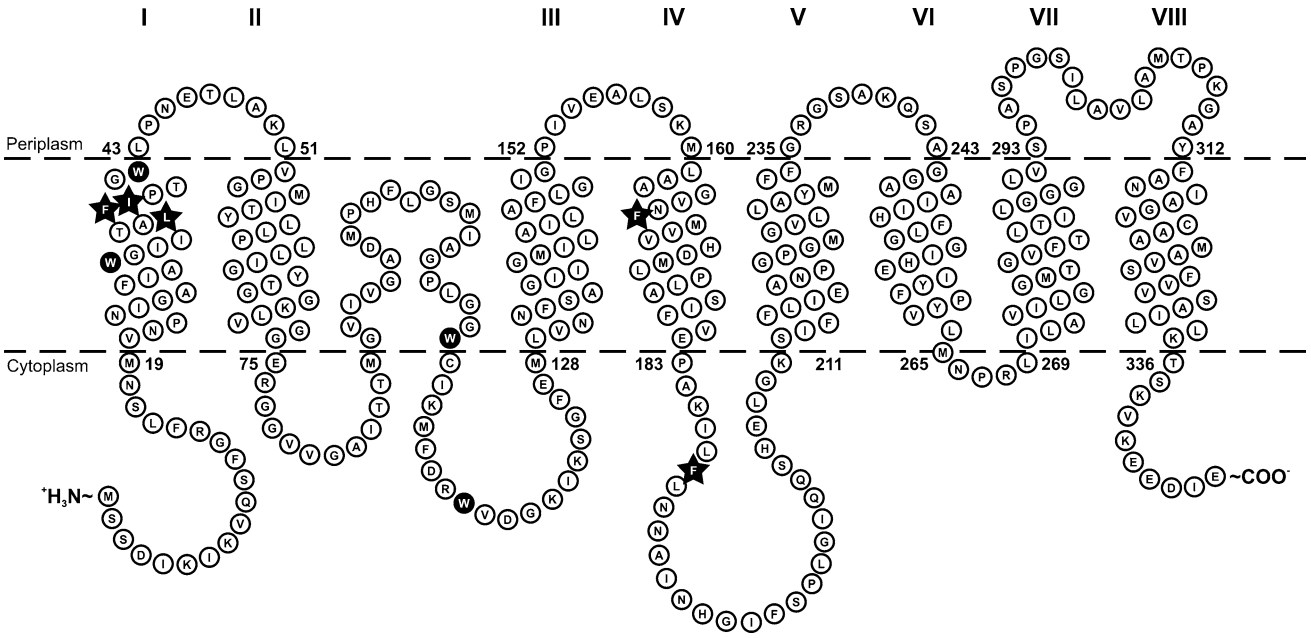

Fig. 1 Topology of wt IIC^{mtl} according to Vervoort et al. 2005. The four Trp positions in the wt protein are highlighted. Trp positions in the single-Trp EII^{mtl} mutants used in this work (W36, W37, W38, W167, and W188) are labeled by a *star*

which the four Trps, all located in IIC^{mtl} (Fig. 1), have been replaced by phenylalanine. Also, numerous single-Trp mutants are available, which have been used in a series of functional and structural studies (Opačić et al. 2010, 2012; Vos et al. 2009a, b).

An attractive feature of EII^{mtl} is the possibility to assay in solution its mannitol-phosphorylation activity and mannitol-binding characteristics (B_{tot}, K_D), which provide detailed information about the functional integrity of the enzyme. For example, the activity of wt EII^{mtl} is essentially the same whether it is embedded in membrane vesicles or solubilized by decylpoly(ethyleneglycol) 300 (C_{10}-PEG) (Veldhuis et al. 2005a). The activity of wt EII^{mtl} is sensitive to the nature of the detergent, the enzyme being strongly (and reversibly) inhibited in the presence of octylglucoside, cholate, and deoxycholate (DOC) (Lolkema et al. 1993). Wt EII^{mtl} harbors one high-affinity binding site per dimer, with a K_D of ~50 nM whether in the membrane-bound state or after solubilization by these detergents. This high affinity is also observed for the TL mutant and, with few exceptions (Opačić et al. 2012; Veldhuis et al. 2005b), for most of the single-Trp mutants characterized to date, whether membrane-bound or solubilized. Thus, replacing the four Trp residues in wt EII^{mtl} by Phe and introducing a Trp somewhere else in the protein does not affect mannitol binding in most of the mutants described. Also, the mannitol-phosphorylation activity in TL and many single-Trp mutants embedded in the native membrane is comparable to that of wt EII^{mtl}. However, some mutants become strongly inhibited when solubilized by detergents like C_{10}-PEG, decylpentaethylene glycolether ($C_{10}E_5$), *n*-dodecyl-*β*-D-maltopyranoside (DDM), or *n*-decyl-*β*-D-maltopyranoside (DM). For example, all constructed single-Trp mutants harboring a Trp in the first putative transmembrane helix of domain C (TMHI) (Fig. 1) show this strong inhibitory effect (Veldhuis 2006), except at positions 30 and 42, which harbor a Trp in the wt protein. The mechanism of this inhibition is not well understood. Because exposure to detergent does not affect the values of K_D and B_{tot}, it has been proposed that the low activity might be due to a perturbation of interdomain phosphoryl group transfer from the A domain, via the B domain, to mannitol bound at the C domain (Veldhuis 2006).

There are indications that APols perturb the structure and function of MPs less than classical detergents do (see e.g., Dahmane et al. 2013; Etzkorn et al. 2013; Liao et al. 2013; Martinez et al. 2002). In the present work, we have examined whether APol A8-35 can be used to keep EII^{mtl} and its detergent-sensitive single-Trp mutants water-soluble without impeding their activity.

Materials and Methods

Chemicals

Amphipol A8-35 (batch PH/FG2) was synthesized as described previously (Gohon et al. 2004, 2006). Fluorescein-labeled A8-35 (FAPol$_{fluo}$) was synthesized as described below. Poly(acrylic acid) (PAA) and 1,4-diazabicyclo[2.2.2]octane

(DABCO) were from Acros, *N,N*-dicyclohexylcarbodiimide (DCI) and fluorescein isothiocyanate (FITC) were from Sigma-Aldrich. Dimethylformamide (DMF) was purchased from SDS (Peypin, France) and used as received. Water ("MilliQ water") was purified on a MilliQ system (Millipore, Saint-Quentinen-Yvelines, France). $C_{10}E_5$- and C_{10}-PEG were obtained from Kwant High Vacuum Oil Recycling and Synthesis (Bedum, The Netherlands). $C_{10}E_5$ was further purified as described previously (Dijkstra et al. 1996). DDM and DM were from Anatrace. KI (Suprapur) was from Merck, Darmstadt. L-Histidine and imidazole (spectroscopic grade) were from Fluka. Bio-Beads SM-2 were from Bio-Rad laboratories. Nitrilotriacetic acid (NTA) resin was from Qiagen. Q-Sepharose was from Amersham Biosciences. D-[1-^{14}C]mannitol (2.07 Gbq mmol^{-1}) was purchased from Amersham Biosciences. Radioactivity measurements were performed using Emulsifier Scintillator Plus obtained from Packard (Groningen, The Netherlands). All other chemicals were of analytical grade.

PAA molecular weight was estimated by size exclusion chromatography in organic medium of the permethylated form of the polymer (see Giusti et al. 2014, for detailed procedure).

Synthesis of Fluorescein-Labeled A8-35 ($FAPol_{fluo}$)

$FAPol_{fluo}$ was derived from an aminated precursor (UAPol) synthesized as described before (Zoonens et al. 2007). Briefly, PAA was modified in *N*-methyl pyrrolidone (NMP) in the presence of dicyclohexylcarbodiimide (coupling reagent), first with octylamine and a selectively monoprotected aminolinker (*N*-benzoyloxy-carbonylethylenediamine), then with iso-propylamine. The three amines were introduced in defined proportions in order to provide the expected A8-35, which bears approximately 4.5 linkers per 40-kDa particle.

The linker was deprotected under mild conditions in methanol in the presence of ammonium formate and palladium on activated charcoal (for a detailed procedure see Zoonens et al. 2007), and then reacted with FITC (4 equivalents) in dry DMF in the presence of DABCO (catalytic amount) at 40 °C for 2 h under inert atmosphere, yielding $FAPol_{fluo}$ (Fig. 2). The product was purified in three steps: (i) by four precipitation cycles in acidic aqueous solution/redissolution at basic pH; (ii) by a 3-day dialysis against Milli-Q water (Spectra/Por dialysis tubing, MWCO = 6–8 kDa); and (iii) by fractionation on a preparative Superose 12 column (Gohon et al. 2006; Zoonens et al. 2007). Fractions were pooled and concentrated to 50 mL under pressure (2 atm) in a Millipore Alphacell-stirred cell (VWR, France). Dialysis and freeze-drying of the resulting solution yielded the purified $FAPol_{fluo}$ in ~50 % yield with respect to UAPol.

Chemical Composition of $FAPol_{fluo}$

The ratios of grafted amines were estimated by ^{1}H and ^{13}C NMR (Gohon et al. 2004; Zoonens et al. 2007). The ratio of grafted dye was estimated by UV–visible spectroscopy using as reference the molar extinction coefficient of fluorescein salt measured in 0.01 M aqueous KOH solution at its maximum absorption (ε_{491} = 87,900 L mol^{-1} cm^{-1}; Seybold et al. 1969) and the molar extinction coefficient of FITC measured in Tris buffer at its maximum absorption (ε_{494} = 86,430 L mol^{-1} cm^{-1}). The extinction coefficients of $FAPol_{fluo}$ measured in Tris buffer in the concentration range 0–7 × 10^{-2} g L^{-1}, were found to be ε'_{491} = 1.84 L g^{-1} cm^{-1} and ε'_{494} = 1.91 L g^{-1} cm^{-1}, corresponding to ~0.9 fluorophores per 40-kDa APol particle (Gohon et al. 2006).

Solution Behavior of $FAPol_{fluo}$ in Aqueous Buffer

The solution behavior of $FAPol_{fluo}$ was characterized by size exclusion chromatography as described (Gohon et al. 2004, 2006). Briefly, a 100-g L^{-1} stock solution of $FAPol_{fluo}$ in water was diluted in Tris buffer and loaded onto a pre-equilibrated and calibrated Superose 12 10-300GL column connected to a FPLC Äkta purifier 10 system. The elution was performed in Tris buffer, and the detection was monitored at 220, 280 and 495 nm.

Expression of EII^{mtl} and Measurement of Its Mannitol-Phosphorylation Activity

The construction of the histidine-tagged single-Trp mutants of the EII^{mtl} and their expression have been reported elsewhere (Opačić et al. 2010; Veldhuis et al. 2005a). Biosynthetic incorporation of 5-fluorotryptophan (5-FTrp) in the single-Trp mutants of EII^{mtl}, when performed, was carried out as described (Broos et al. 2003). Inside-out (ISO) vesicles from *E. coli* inner membrane were prepared as described (Broos, ter Veld and Robillard 1999). Activity assays were performed as described (Robillard and Blaauw 1987).

Preparation of Detergent-Solubilized and APol-Trapped EII^{mtl}

E. coli ISO vesicles with the single-Trp mutants of EII^{mtl}, W36, W37, or W38 (containing a single Trp in the TMHI at positions 36, 37, and 38, respectively, see Fig. 1) were solubilized by adding the vesicles slowly, under stirring, to 19 volumes of 20 mM Tris/HCl, pH 8.4, 50 mM NaCl, 10 mM β-mercaptoethanol and either 0.5 % purified deoxycholate (DOC) (Dijkstra et al. 1996) or 0.5 % C_{10}-PEG. After 20 min at room temperature, A8-35 was added

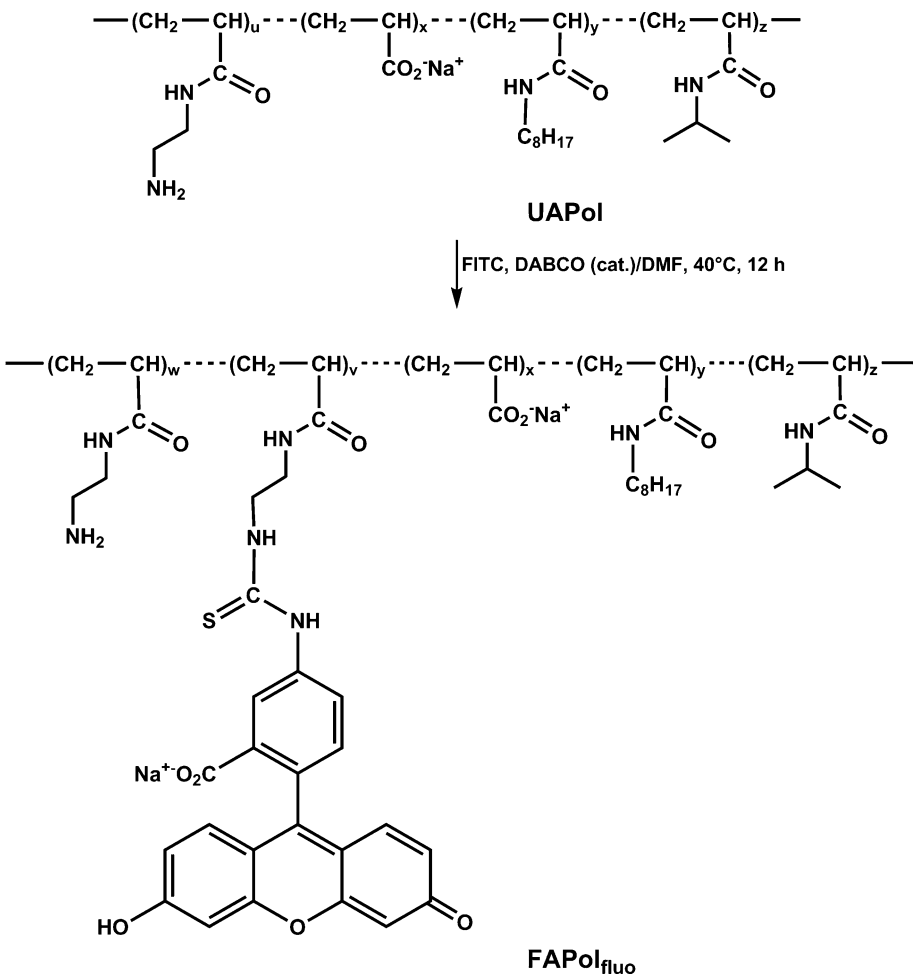

Fig. 2 Synthesis of $FAPol_{fluo}$ by derivatization of UAPol (A8-35 carrying an amino arm; see Zoonens et al. 2007). Reactive fluorescein (FITC) was used in 4× molar excess over free amino functions, whereas DABCO was used in catalytic amount (1:10 molar ratio with respect to amino functions). The reaction was carried out in DMF at 40 °C for 12 h. Percentage of the various moieties (randomly distributed): UAPol: $x \approx 41.5$ %, $y \approx 23$ %, $z \approx 34$ %, $u \approx 1.5$ %; $FAPol_{fluo}$: $x \approx 41.5$ %, $y \approx 23$ %, $z = 34$ %, $v \approx 1.25$ % and $w \approx 0.25$ %. Each chain contains on average ~35 units (see Giusti et al. 2014)

in a 50:1 mass ratio relative to EII^{mtl}. After 15-min incubation at 0 °C, the mixture was diluted 40× in 20 mM Tris/HCl, pH 8.4, 200 mM NaCl, 5 mM dithiothreitol (DTT), and left at 0 °C for 2.5 h, after which samples were centrifuged for 20 min at 230,000×*g*. In control experiments, done for each of the three mutants, the same procedure was followed except that neither detergent nor APol was added and the samples were not centrifuged (see Scheme in Fig. 3). For activity measurements, supernatants were diluted 10× in the assay buffer, containing neither detergent nor APol. The activity was measured using 60 µM [^{14}C] mannitol.

Purification and "On-Column" APol Trapping Procedure for the W36 EII^{mtl} Mutant Solubilized by DOC

A 1-mL aliquot of W36 EII^{mtl} ISO vesicles was solubilized by adding it slowly, under stirring, to 19 mL of 20 mM Tris/HCl, pH 8.4, 0.5 % DOC, 50 mM NaCl, 20 mM imidazole, 100 µM mannitol, 10 mM β-mercaptoethanol. After 30 min at 25 °C, the sample was centrifuged at 350,000×*g* at 4 °C for 20 min and the supernatant mixed with 1 mL Ni–NTA slurry and incubated for 1 h at 4 °C. The resin was poured in a column and washed with 5-mL buffer containing 20 mM Tris/HCl, pH 8.4, 0.25 % DOC, 50 mM NaCl, 20 mM imidazole, 100 µM mannitol, 2 mM reduced glutathione. 1 mL of the same buffer, but containing 50 mM NaCl, and 3.1 g L^{-1} A8-35 was added to the resin. After 20 min incubation, the suspension was mixed with 9 mL of the latter buffer, but without DOC, and the suspension was rotated overnight at 4 °C. The protein was eluted using a buffer containing 20 mM Tris/HCl, pH 8.2, 50 mM L-histidine and 2 mM reduced glutathione. Eluted fractions were analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with Coomassie Brilliant Blue (CBB).

Purification and "Off-Column" APol Trapping Procedure for the Tryptophan-Less EII^{mtl} (TL-EII^{mtl}) and Single-Trp Mutants of EII^{mtl}

TL-EII^{mtl} and mutants W30, W36, and W38 were used for phosphorylation activities studies, whereas 5-FTrp

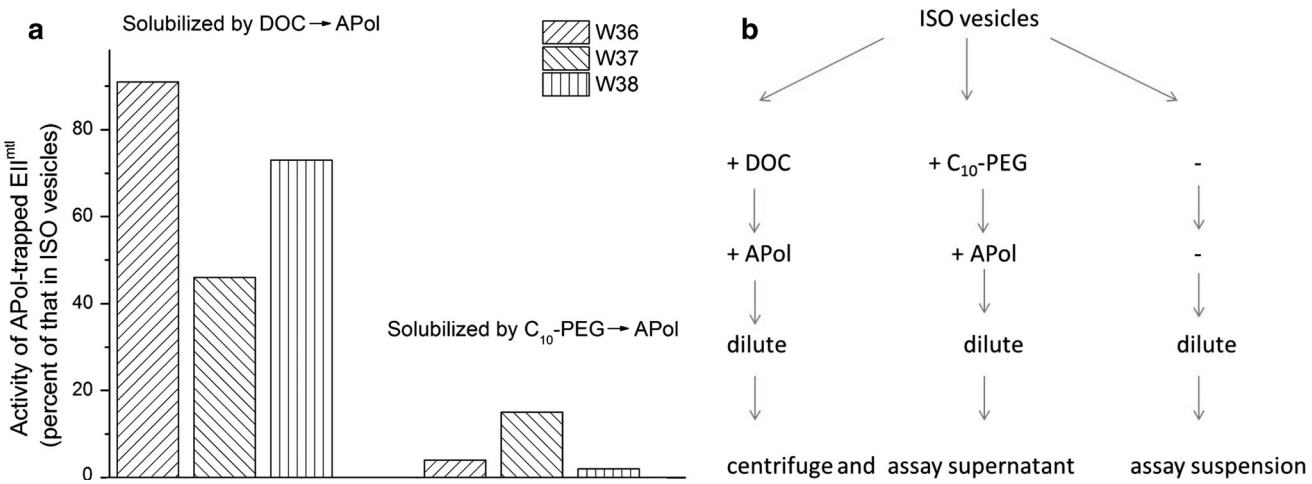

Fig. 3 Mannitol-phosphorylation activities of EII^{mtl} mutants W36, W37, and W38 trapped by A8-35. **a** Mannitol-phosphorylation activities in supernatants after centrifugation of W36, W37, and W38 samples extracted from membranes by either deoxycholate (*left*) or C_{10}-PEG (*right*) and transferred to A8-35, relative to their activity in unsolubilized ISO vesicles. **b** Experimental outline used to obtain the samples assayed in **a**. See "Materials and Methods" section for experimental details

containing mutants W167 5F and W188 5F were used for fluorescence spectroscopy studies. The isolation procedure was essentially the same as presented above for W36 EII^{mtl} up to and including the washing of the Ni–NTA resin with DOC buffer. The DOC detergent was exchanged to 0.5 mM DDM by successive rinsing of the column with 5 column volumes of the following three buffers: (i) 20 mM Tris/HCl, pH 7.6, 0.5 mM DDM, 150 mM NaCl, 10 mM imidazole, 1.5 mM reduced glutathione; (ii) same but with 20 mM imidazole; and (iii) same but with 30 mM imidazole. The protein was eluted with 20 mM Tris pH 7.6 buffer containing 1.5 mM reduced glutathione, 0.5 mM DDM, and 50 mM L-histidine. NaCl, up to a final concentration of 100 mM, was added to the eluted fractions. Mutants used for the activity assays only were trapped with A8-35 at this stage.

Mutants used for the fluorescence spectroscopy studies were further purified using a Q-Sepharose column. The protein was eluted with 350 mM NaCl in 20 mM Tris–HCl, pH 8.4, 1 mM reduced glutathione, and 0.5 mM DDM. The exchange with unlabeled A8-35 or FAPol was carried out by first diluting the sample of purified mutant 2× with buffer containing 20 mM Tris–HCl, pH 8.4, 1 mM reduced glutathione, and 0.5 mM DDM, to reduce the amount of NaCl to 175 mM (this step was omitted if trapping was done immediately after the Ni–NTA purification). Then, the sample was mixed with A8-35 or FAPol at 1:2–1:5 protein to APol (or FAPol) mass ratio. The mixture was incubated at 4 °C for 50 min, Bio-Beads SM-2 were added in a mass ratio of 1:10 detergent/Bio-Beads, and the mixture incubated overnight at 4 °C. After removal of the Bio-Beads, the samples were ultracentrifuged (250,000×*g*, 20 min) and the supernatant used for fluorescence spectroscopy studies.

Fluorescence Spectroscopy Measurements

Fluorescence emission spectra were collected at ~23 °C using a PTI International type C60/C-60 SE fluorometer, with the excitation and emission slit widths set at 2 and 5 nm, respectively. The excitation wavelength was set at 295 nm and emission collected from 305 to 460 nm. All spectra were recorded at least twice and were corrected for background emission and for instrument response. In KI quenching experiments, protein samples, either in 0.5 mM DDM or trapped with A8-35, were titrated with increasing concentrations of KI using a KI stock solution.

Results and Discussion

EII^{mtl} trapped in APol A8-35 was prepared according to two protocols: either the detergent was substituted for APol in solution, as is classically done (Zoonens et al. 2014), or substitution was carried out while His-tagged EII^{mtl} was immobilized onto a Ni–NTA column.

Wild-Type, Trp-Less and Single-Trp Mutants of EII^{mtl} are Soluble and Active Following Trapping in Solution with Amphipol A8-35

The single-Trp mutants W36, W37, and W38, mutated at positions predicted to lie in the first putative TMH of domain C (Fig. 1), are inactive in detergent solutions

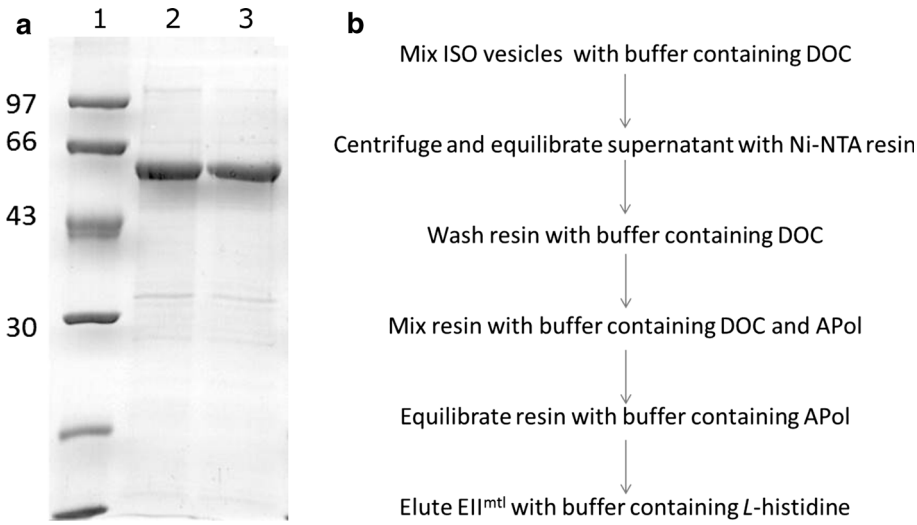

Fig. 4 Purification of APol-trapped EII^{mtl} mutant W36. **a** SDS-PAGE analysis of APol-trapped mutant W36 purified on a Ni–NTA column. *First lane* molecular markers with indicated masses in kDa; *second* and *third lanes* the two most enriched fractions eluted from the Ni–NTA column with a buffer containing 50 mM L-Histidine. **b** Outline of the experimental protocol used to obtain the W36 samples shown in **a**

(Veldhuis 2006). As APols as a rule are not dissociating enough to extract MPs from lipid bilayers, inside-out (ISO) vesicles containing these mutant proteins were first mixed with 0.5 % DOC, a well-established procedure to extract EII^{mtl} (Broos et al. 1999; Robillard et al. 1993). In another series of experiments, 0.5 % C_{10}-PEG was used for extraction, whereas in a third, control series of experiments, no detergent was added. A8-35, at an APol/EII^{mtl} mass ratio of ~50, was added to each of the detergent-solubilized sample, after which the samples were diluted 200× in surfactant-free buffer, so that the final concentration of each detergent fell below its critical micellar concentration (cmc) (~25× below cmc for DOC, ~15× below cmc for C_{10}-PEG) (see Scheme in Fig. 3). Following dilution, the protein finds itself surrounded, and kept water-soluble, by nearly pure A8-35. Indeed, because detergents and APols mix nearly ideally (Tribet et al. 2009; Zoonens et al. 2007), it is expected that, in a solution where the concentration of free detergent is ~1/20 its cmc, it represents, in mass, ~1/20 of the surfactant layer surrounding the protein. After equilibration, the solution was centrifuged and the mannitol-phosphorylation activity in the supernatant determined. For the W36 mutant, extracted from membranes by DOC and transferred to A8-35, a high phosphorylation activity was found, which was only ~10 % lower than that of the non-centrifuged sample devoid of detergent (control series) (Fig. 3). Mutants W37 and W38 exhibited, respectively, a ~54 and ~27 % lower activity. In contrast, the mutants that had been extracted from membranes by C_{10}-PEG, before being transferred to A8-35, were found to be almost inactive (Fig. 3). These experiments suggest that A8-35 is an attractive medium in which to keep these mutants soluble, provided they are extracted from the membrane using DOC rather than C_{10}-PEG.

Purification and Amphipol-Trapping of Column-Immobilized EII^{mtl}

The possibility to isolate EII^{mtl} using A8-35 was explored next with mutant W36. In this approach, purification and trapping were carried out after immobilizing the detergent-solubilized protein on a Ni–NTA column, rather than in solution as described in the previous section. W36 EII^{mtl} was extracted from ISO vesicles with DOC. After centrifugation, the supernatant was mixed with Ni–NTA resin. Unbound proteins were eluted with buffer containing 0.25 % DOC, and the resin subsequently mixed with the same buffer containing 3 g A8-35 per g EII^{mtl}. The sample was diluted after 20 min to bring the DOC concentration ~5× below its cmc. After equilibration, the resin was washed with surfactant-free buffer and EII^{mtl} eluted with 50 mM L-Histidine. A quite pure protein, kept soluble by A8-35, can be isolated in this way (Fig. 4).

Preparation of Amphipol-Trapped EII^{mtl} Samples for Fluorescence Spectroscopy

Low-molecular weight fluorescent impurities in EII^{mtl} samples can be removed efficiently by anion-exchange chromatography (Dijkstra et al. 1996). However, the presence of many carboxylate groups in A8-35 results in a tight binding of EII^{mtl}/A8-35 complexes to cationic supports, prohibiting their elution. Therefore, EII^{mtl} was isolated first in detergent solution using the standard isolation protocol, which consists of two column steps, metal chelating chromatography and anion-exchange chromatography (Broos et al. 2000; Vos et al. 2009a). In this case, the eluted protein is solubilized by a neutral detergent like $C_{10}E_5$, DM, or DDM, and the detergent replaced by A8-35 only after the protein has been eluted from the second

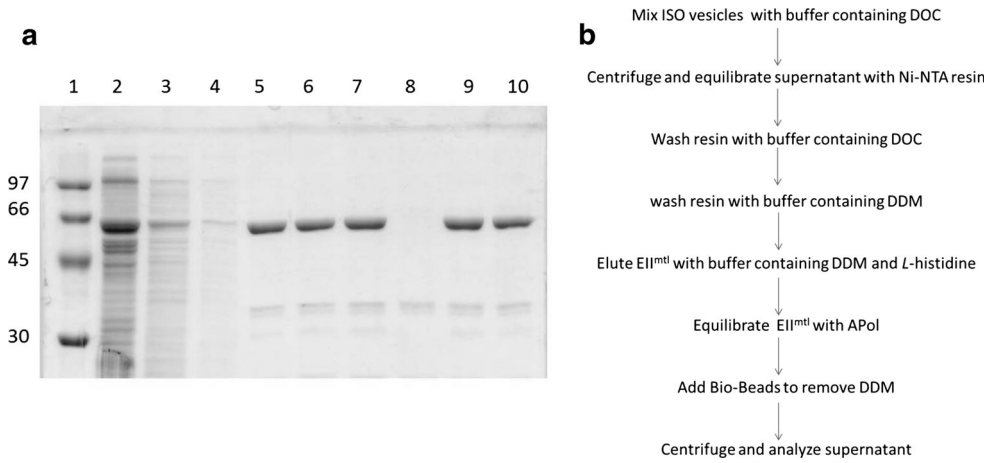

Fig. 5 Purification of APol-trapped Tl EII^{mtl}. **a** SDS-PAGE analysis of samples at different stages of the purification procedure. *Lane 1* molecular markers with indicated mass in kDa; *2* solubilized vesicles; *3* Flow-through of Ni–NTA column; *4* First wash; *5* A8-35-trapped, non-centrifuged sample, *6* A8-35-trapped, ultracentrifuged sample, supernatant; *7* sample in 0.5 mM DDM after overnight incubation with Bio-Beads, non-centrifuged; *8* Sample in 0.5 mM DDM after overnight incubation with Bio-Beads, ultracentrifuged, supernatant; *9* Sample in 0.5 mM DDM, non-centrifuged; *10* Sample in 0.5 mM DDM, ultracentrifuged, supernatant. **b** Outline of the experimental protocol used to obtain the TL samples shown in **a**

column (see Scheme in Fig. 5 and "Materials and Methods" for details). TL and several single-Trp-containing EII^{mtl} mutants were used to explore this purification scheme using DDM as the detergent. Purified samples were trapped in APol by supplementing them with A8-35 and removing the DDM with Bio-Beads. Samples at different stages of the isolation procedure were analyzed by SDS-PAGE (Fig. 5).

Comparison of the APol-trapped TL EII^{mtl} sample before and after centrifugation (Fig. 5, lane 5 vs. lane 6) shows that the protein is efficiently kept soluble by A8-35. In contrast, if no APol was added to DDM-solubilized TL EII^{mtl}, mixing with Bio-Beads followed by ultracentrifugation resulted in pelleting all of the protein (compare lanes 8 vs. 7).

Selected TL EII^{mtl} samples (Fig. 5, lanes 5–10) were assayed for their phosphorylation activity. The activity data (Fig. 6) show that, at comparable concentrations, as estimated by SDS-PAGE, TL EII^{mtl} is $\sim$2.5$\times$ more active as a complex with A8-35 than it is in the presence of 0.5 mM DDM (compare Fig. 6a, c).

The same result was obtained for W30 EII^{mtl}: This mutant also showed a 2.5–3$\times$ increase in activity after transfer from DDM to A8-35 (*not shown*). However, two other EII^{mtl} mutants known to be very sensitive to DDM, W36 and W38, could not be reactivated by transfer to A8-35 (*data not shown*). As pointed out before, given that the K_D and B_{tot} parameters for mannitol binding are not affected by detergents (Veldhuis 2006), the inhibition of mannitol-phosphorylation activity is believed to be related to a blockage of the transfer of phosphate from IIA^{mtl}, via IIB^{mtl}, to IIC^{mtl}-bound mannitol. Whatever is interfering

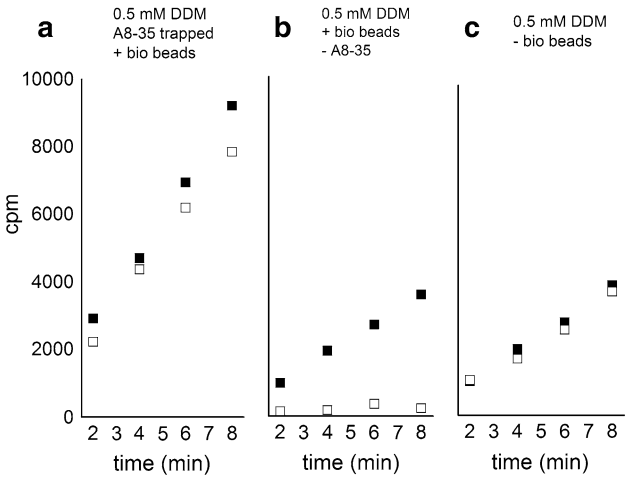

Fig. 6 Phosphorylation activity assays of three preparations of TL EII^{mtl}. **a** APol-trapped TL EII^{mtl}, prepared by supplementing a solution in DDM with A8-35 and removing the detergent by incubation with Bio-Beads (lane 6 in Fig. 4). **b** A TL EII^{mtl} sample depleted of DDM with Bio-Beads in the absence of A8-35 (lane 8 in Fig. 5). **c** Another aliquot of the same sample as in **b**, kept in DDM (lane 10 in Fig. 4). *Solid symbols* correspond to non-centrifuged samples, *open symbols* to the supernatant of the same samples after centrifuging them at 200,000$\times g$ for 20 min. TL EII^{mtl} was isolated according to the scheme shown in Fig. 5b. 1 mM [^{14}C]mannitol was used in the mannitol-phosphorylation assay

with this transfer (*e.g.*, the loss of critical lipids), it is obviously reversible in mutant W30 and not in W36 or W38. It is worth noting that the recovery of membrane-like transitions in the late photocycle of BR upon transfer from detergent to APols has been traced to the rebinding of lipids (Dahmane et al. 2013), and that the same mechanism

is suspected in the case of the acetylcholine receptor (Martinez et al. 2002). Controlling the loss of lipids, e.g., by resorting to DOC rather than DDM for the solubilization step, or by carrying out the solubilization and purification steps under milder conditions and/or in the presence of lipids, may be one route to identify conditions that preserve the activity of all APol-trapped EII^{mtl} mutants.

Use of Amphipol-Trapped EII^{mtl} for Fluorescence Measurements

Functional assays are a powerful means to investigate the impact of a detergent or APol on the MP it solubilizes. Information about the effect of surfactants on MP structure are harder to come by, but can be obtained by monitoring the fluorescence properties of single-Trp-containing proteins. Insight about the location of the Trp residue with respect to the transmembrane region, onto which the APol is adsorbed, can be obtained using Förster resonance energy transfer (FRET) between Trp and a fluorescent APol (FAPol). In previous studies, FRET between an APol labeled with NBD (7-nitrobenz-2-oxa-1,3-diazol-4-yle) and the Trp residues of tOmpA, the transmembrane domain of outer membrane protein A from *E. coli*, has been exploited to monitor surfactant exchange at the surface of tOmpA (Zoonens et al. 2007). For the present work, a fluorescein-labeled version of A8-35 ($FAPol_{fluo}$) has been synthesized (see "Materials and Methods" Section), fluorescein presenting the advantages of a higher quantum yield and better stability than NBD. As unlabeled A8-35 (Gohon et al. 2006), $FAPol_{fluo}$ assemble into well-defined particles, which are free of unbound fluorescein (Fig. 7a). As

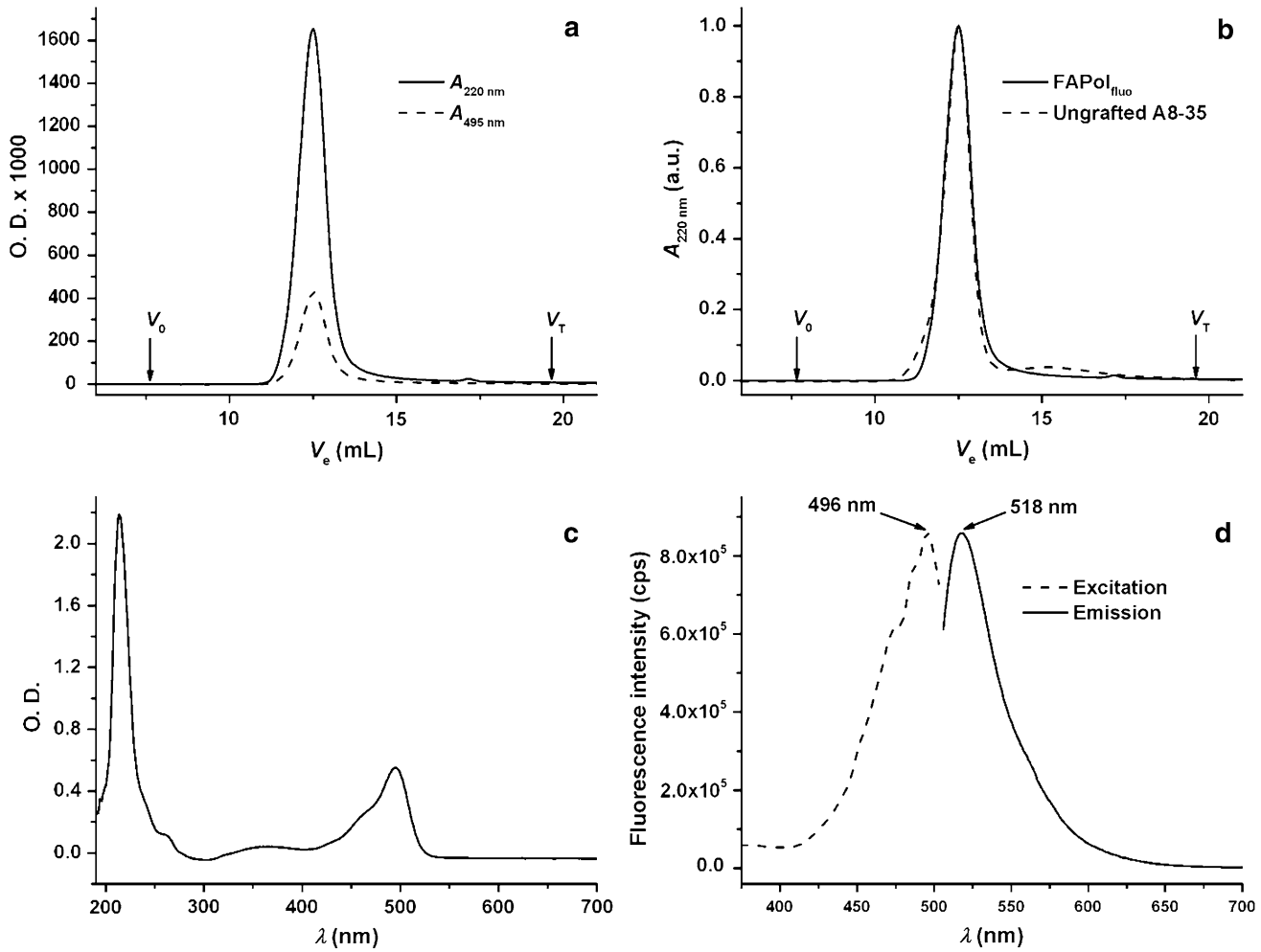

Fig. 7 Solution and spectral properties of $FAPol_{fluo}$. **a** Elution profiles at 220 nm (*solid line*) and 495 nm (*dashed line*). The concentration injected was 10 g L^{-1}. Elution buffer: 0.1 M NaCl, 20 mM Tris/HCl, pH 8. V_e, elution volume, V_0, excluded volume, V_T, total volume. **b** Superimposition of the elution profiles of $FAPol_{fluo}$ (*solid line*) and ungrafted A8-35 (*dashed line*), obtained under the same conditions, with the detection monitored at 220 nm. The profiles were normalized to the same maximum. a.u.: arbitrary units. **c** UV–visible absorption spectrum of a 0.66-g L^{-1} solution of $FAPol_{fluo}$ in the same buffer. **d** Emission (*solid line*) and excitation (*dashed line*) spectra of a 0.001-g L^{-1} solution of $FAPol_{fluo}$ in the same buffer

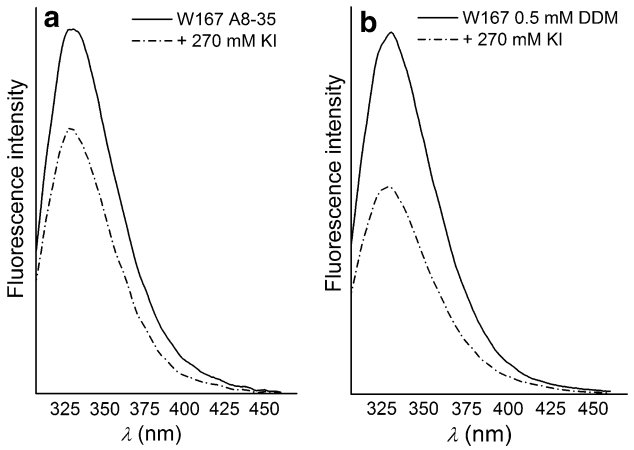

Fig. 8 Emission spectra of mutant W167 5F in the absence and in the presence of 270 mM KI. **a** Spectra of the mutant trapped in A8-35. **b** Spectra of the mutant in 0.5 mM DDM

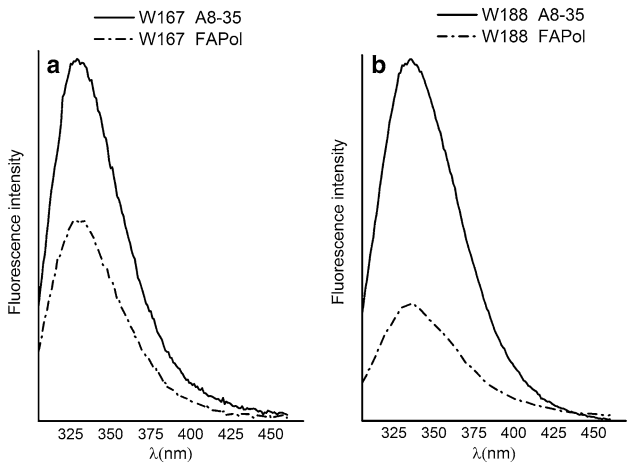

Fig. 9 Emission spectra of EII^{mtl} mutants. **a** W167 5F and **b** W188 5F in APol-trapped versus FAPol-trapped forms

observed before for other FAPols (reviewed in Le Bon et al. 2014), the presence of a relatively small label attached to the ~40-kDa APol particles does not affect their solution behavior, A8-35 and $FAPol_{fluo}$ particles migrate identically upon SEC (Fig. 7b). The spectroscopic properties of $FAPol_{fluo}$ are shown in Fig. 7c (extended UV–visible absorption spectrum) and 7d (fluorescence excitation and emission spectra). Because the absorbance bands of fluorescein overlap the emission bands of Trp or 5-fluoro-tryptophan (5-FTrp), trapping a MP with $FAPol_{fluo}$ results in FRET, which provides information about the distance between the two partners. Analysis of a family of single-Trp mutants trapped by $FAPol_{fluo}$ can, in principle, provide precious information about the relative position of each Trp with respect to the membrane environment, and how it may change during the functional cycle of the protein. A first test of the feasibility of this approach has been carried out here.

The environment of positions 167 (mutant W167) and 188 (mutant W188) in the transmembrane domain of EII^{mtl}, IIC^{mtl} (Fig. 1), was studied using this approach, using mutants labeled with 5-FTrp. This isosteric analog of Trp has a red-shifted absorption, compared to Trp, and its fluorescence generally exhibits a monoexponential decay when incorporated into proteins, greatly simplifying the collection and interpretation of time-resolved fluorescence data. Important for the steady-state fluorescence experiments presented here is the very low sensitivity of the quantum yield of 5-FTrp to changes in its microenvironment (Broos et al. 2004; Liu et al. 2005). Thus, a conformational change in EII^{mtl} around this probe is not expected to yield a large, if any, change in emission intensity, as is typically observed for Trp. This makes it attractive to investigate the solvent accessibility of 5-FTrp, using a water-soluble quencher like KI, or as a donor in FRET experiments, because changes in 5-FTrp emission are directly related to its interaction with the quencher or acceptor, respectively.

Quenching by KI of the fluorescence of EII^{mtl} containing a single 5-FTrp at position W167 was studied either in DDM solution or after trapping by A8-35. The extent of fluorescence quenching by KI is surfactant-dependent (Fig. 8). The decreased level of quenching observed with the APol-trapped protein upon addition of KI indicates a diminished accessibility, which can be a consequence either of repulsive electrostatic interactions between COO^- groups of A8-35 and I^- ions, or from a slightly different conformation or dynamics of the protein.

Fluorescence emission intensities of mutants W167 5F and W188 5F in plain A8-35- versus $FAPol_{fluo}$-trapped form are shown in Fig. 9. The comparison of Figs. 8 and 9a shows that W167 is sensitive both to a water-soluble quencher and to an APol-carried one, suggesting an interfacial position, whereas the comparison between Fig. 9a and b suggests that W167 lies farther away from the APol layer than W188. Whereas these data are preliminary, they do suggest that FAPols can be useful tools for exploring the topology of MPs.

Conclusion and Outlooks

In this work, procedures are presented for the isolation of pure, water-soluble, and functional EII^{mtl}, trapped by A8-35. They make it possible to isolate EII^{mtl}, including detergent-sensitive EII^{mtl} mutants, with high mannitol-phosphorylation activity and a level of purity suitable for fluorescence studies. This is the first demonstration of isolating these detergent-sensitive EII^{mtl} mutants in active form. A large number of $C_{10}E_5$-solubilized single-Trp EII^{mtl} mutants have been characterized in detail using

fluorescence and/or phosphorescence spectroscopy. Collecting similar data for a broader range of mutants trapped in APols should help understanding outstanding issues about APol/MP interactions like the "dampening" effect of APol on MP dynamics—an effect that could be investigated by measuring such spectroscopic parameters as Trp phosphorescence lifetimes or the rotational mobility of the Trp side chains. It would be interesting as well to use APol-trapped EII^{mtl} single-Trp mutants to further examine whether quenching by appropriately functionalized APols versus soluble quenchers can provide information about the topology of $EIIC^{mtl}$, and of MPs in general, as well as about conformational changes during the functional cycle.

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