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Structural investigation of the transmembrane C domain of the mannitol permease from *Escherichia coli* using 5-FTrp fluorescence spectroscopy

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ABSTRACT

The mannitol transporter EII^{mtl} from *Escherichia coli* is responsible for the uptake of mannitol over the inner membrane and its concomitant phosphorylation. EII^{mtl} is functional as a dimer and its membrane-embedded C domain, IIC^{mtl}, harbors one high affinity mannitol binding site. To characterize this domain in more detail the microenvironments of thirteen residue positions were explored by 5-fluorotryptophan (5-FTrp) fluorescence spectroscopy. Because of the simpler photophysics of 5-FTrp compared to Trp, one can distinguish between the two 5-FTrp probes present in dimeric IIC^{mtl}. At many labeled positions, the microenvironment of the 5-FTrps in the two protomers differs. Spectroscopic properties of three mutants labeled at positions 198, 251, and 260 show that two conserved motifs (Asn194-His195 and Gly254-Ile255-His256-Glu257) are located in well-structured parts of IIC^{mtl}. Mannitol binding has a large impact on the structure around position 198, while only minor changes are induced at positions 251 and 260. Phosphorylation of the cytoplasmic B domain of EII^{mtl} is sensed by 5-FTrp at positions 30, 42, 251 and 260. We conclude that many parts of the IIC^{mtl} structure are involved in the sugar translocation. The structure of EII^{mtl}, as investigated in this work, differs from the recently solved structure of a IIC protein transporting diacetylchitobiose, ChbC, and also belonging to the glucose superfamily of EII sugar transporters. In EII^{mtl}, the sugar binding site is more close to the periplasmic face and the structure of the 2 protomers in the dimer is different, while both protomers in the ChbC dimer are essentially the same.

1. Introduction

The EII sugar transporters of the bacterial-specific phospho*enol*pyruvate (PEP) dependent phosphotransferase system (PTS) phosphorylate their cognate substrates during transport over the membrane. EII transporters belong to the class of group translocation transporters, which is distinct from the classes of primary and secondary transporters [1]. The D-mannitol specific mannitol permease from *Escherichia coli*, EII^{mtl}, is one of the best-characterized EII sugar transporters [2]. It is a 68-kDa protein composed of three covalently linked domains, two cytoplasmic domains, A and B, and a membrane-embedded C domain (IIC^{mtl}). It transports mannitol from the periplasm to the cytoplasm, where it is released as mannitol-1-phosphate [2,3]. The phosphoryl group originates from PEP and is delivered to mannitol in the C domain *via* His^{554} in the A domain and Cys^{384} in the B domain of EII^{mtl}, respectively. The structures of the A and the B domains have been solved by X-ray crystallography and NMR spectroscopy [4,5]. For the membrane-embedded IIC^{mtl} (346 residues), which harbors the mannitol translocation pathway, a 2D projection map at 5 Å resolution is available [6]. EII^{mtl} is functional as a dimer and it contains one high-affinity mannitol binding site per dimer [7].

Various biophysical and biochemical approaches, including Trp fluorescence spectroscopy, have been exploited to study mechanistic and structural features of EII^{mtl} [8–11]. WT EII^{mtl} contains four Trp residues, all located in the C domain but they can be replaced by Phe residues without significant loss of function [8]. This mutant protein was used to construct single Trp mutants of EII^{mtl} [12,13]. Recently, 5-fluoroTrp (5-FTrp), which displays much more homogeneous fluorescence decay kinetics than Trp [14–16], was biosynthetically incorporated into these mutants. In favorable cases, one can distinguish between the two fluorophores present per dimer and it was found that dimeric EII^{mtl} does not form a symmetric dimer [11]. Förster resonance energy transfer (FRET) measurements carried out with 19 single Trp mutants of EII^{mtl}, where 5-FTrp was the donor and the substrate analogue azi-mannitol the acceptor, indicated that the mannitol binding site is

Abbreviations: EII^{mtl}, the mannitol-specific transporting and phosphorylating enzyme from *E. coli*; IIC^{mtl}, membrane-embedded C domain of EII^{mtl}; Trp, tryptophan; 5-FTrp, 5-fluorotryptophan; TOE, tryptophan octyl ester: mannitol, D-mannitol; FRET, Förster resonance energy transfer; FWHH, full width at the half height; ϕ, rotational correlation time; NATA, N-acetyl-tryptophanamide; PEG, Polyethylene glycol

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Nomenclature

Trp-less EII^{mtl} indicates wt EII^{mtl} in which the four native Trps are replaced by Phe. W30, W38, W42, W66, W147, W167, W180, W188, W198, W251, W260, W282, and W327 are the single-Trp-containing EII^{mtl} mutants based on Trp-less EII^{mtl}.
W30 C384S, W42 C384S, W251 C384S, and W260 C384S are the single-Trp-containing EII^{mtl} mutants based on Trp-less EII^{mtl} carrying a Cys384Ser mutation.

asymmetrically positioned in the dimer [12]. Residue positions 30, 38, 97, 133 were found at 7–8 Å from the mannitol-binding site. Furthermore, experiments with stably phosphorylated mutants showed that this site is also the site where mannitol becomes phosphorylated.

Here we characterize 13 positions in the C-domain of EII^{mtl} by 5-FTrp fluorescence spectroscopy, including two native Trp positions at 30 and 42. Conservative Phe→Trp and Tyr→Trp mutations were introduced in Trp-less EII^{mtl} at positions spread through the C domain (positions 66, 147, 167, 180, 188, 198, 251, 260, 282, and 327). Ile38, close to the mannitol binding site was also mutated to Trp. Characterization of these mutants in absence and presence of mannitol informs which parts of IIC^{mtl} are involved in the mannitol transport process and the 5-FTrp spectroscopy approach can also elucidate structural differences between the two protomers in dimeric EII^{mtl}. Recently, it was reported that mutants containing the C384S mutation in the B domain become irreversibly phosphorylated at Ser384, mimicking therefore the activated form of the enzyme [17,18]. Here 5-FTrp fluorescence spectroscopy is used to investigate if the IIC^{mtl} structure near positions 30, 42, 251, and 260 is affected by B domain phosphorylation. Positions 30 and 42 are close to the mannitol binding site while positions 251 and 260 flank the conserved GIXE motif (Gly254-Ile255-His256-Glu257). The presented data provide structural details of EII^{mtl} near the 5-FTrp labeled positions and the impact of mannitol binding and B domain phosphorylation on this. Moreover, the data informs at which 5-FTrp labeled positions the two protomers in dimeric EII^{mtl} differ in structure. In the course of this work, the first 3D structure of a IIC domain was presented, the diacetylchitobiose transporter, ChbC, from *Bacillus cereus* [19]. Comparison of the structural features of EII^{mtl} with the ChbC structure shows the single high affinity mannitol binding site in EII^{mtl} is located more close to the periplasmic face than the two binding sites present in ChbC, both binding diacetylchitobiose. Another clear difference is that both protomers in dimeric EII^{mtl} differ in structure while in dimeric ChbC the structure of both protomers are essentially the same.

2. Materials and methods

2.1. Chemicals and reagents

$C_{10}E_5$ detergent was obtained from Kwant High Vacuum Oil Recycling and Synthesis, Bedum, The Netherlands, and fluorescent impurities were removed as described [20]. KI (Suprapur) was from Merck. N-decyl-β-D-maltoside (DM) was from Anatrace.

2.2. EII^{mtl} mutants

The construction of the single-Trp-containing mutants of EII^{mtl} used in this work have been described before [12,13,21]. Briefly, all single Trp mutants, except W38, were based on Trp- less EII^{mtl} with a N-terminal His_6 tag [13] while mutant W38 is based on Trp- less EII^{mtl} with a C-terminal His_6 tag [12]. Trp codons were introduced using the QuickChange site-directed mutagenesis kit of Stratagene. The nonvectorial phosphorylation activities and the mannitol dissociation constants (K_Ds) of the mutants have been reported before [8,12,21]. The nonvectorial phosphorylation activities of the investigated mutants varied from 65% of the WT EII^{mtl} activity (mutants W180 and W282) to 15% in the case of mutants W251 and W327 [7]. As expected, mutants containing the C384S mutation were inactive [22]. The C384S mutation does not affect the K_D of mannitol [23]. The K_Ds of the mutants are presented in Table S1.

Biosynthetic incorporation of 5-FTrp in the single Trp mutants of EII^{mtl} was according to a published procedure [24]. This procedure results in over 95% incorporation of 5-FTrp at a Trp codon position [24]. In-side-out vesicles were prepared as described [10]. EII^{mtl} mutants were purified using a two-step purification procedure [11]. The C384S mutants were first phosphorylated and subsequently purified as described [12]. Mass spectrometry analysis showed that this procedure resulted in complete phosphorylation of Ser384 in these mutants [12]. For this analysis, the purified C384S mutant was treated with trypsin and analyzed by nano-flow reversed-phase high performance liquid chromatography (RP-HPLC) tandem mass spectrometry (MS/MS) on a linear quadrupole ion trap-Fourier transform hybrid mass spectrometer (LTQ Orbitrap XL). The 380–390 peptide was detected exclusively in the phosphorylated form, with a deviation of 0.003 Da from the theoretical mass. MS/MS experiments identified Ser384 as the phosphorylation site.

2.3. Fluorescence experiments

All fluorescence spectroscopy measurements were performed in buffer containing 20 mM Tris, pH 8.4, 1 mM reduced glutathione, 250 mM NaCl and 0.25% (*v*/*v*) $C_{10}E_5$. In this optimized buffer system, EII^{mtl} is present as dimers [2] and the specific mannitol phosphorylation activity of purified wt EII^{mtl} is only 10–20% lower compared to the unpurified protein in *E. coli* membrane vesicles [7,25]. For steady state fluorescence measurements a Fluorolog3-22 spectrofluorometer (Jovin Yvon) was used. The excitation wavelength was 295 nm and the emission was measured from 305 to 500 nm. The excitation and emission slit widths were 1.25 and 4 nm respectively, and the temperature was 23 °C. All spectra were recorded at least twice and were corrected for background emission and instrument response. The emission intensities presented in Stern–Volmer (S–V) plots were calculated using the integrated area of the emission peaks between 305 and 460 nm. If both 5-FTrp residues show a similar solvent accessibility towards KI, a linear Stern–Volmer (S–V) dependence is expected. If the solvent-accessibility of the two 5-FTrp probes is not the same, an S–V plot, featuring a downward curvature, is obtained [26]. KI and not acrylamide was used as quencher because the latter likely partitions into the $C_{10}E_5$ belt surrounding EII^{mtl}, in this way biasing the quenching data. Moreover, acrylamide can quench the singlet Trp state quite efficiently via a through space mechanism [27].

Time resolved fluorescence spectroscopy measurements were performed at 20 °C as described elsewhere [11]. The excitation wavelength was 305 nm and a 368 nm interference filter (IF) with a 17 nm full width at half height (FWHM) band-pass (Schott) was used. In time-correlated single photon counting measurements, where excitation was at 295 nm, a 349 nm IF with a 8 nm FWHH band pass filter and a cut-off filter (WG320, Schott) were used to collect the emission [14]. An excitation wavelength of 295 nm was used for mutants