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Domain complementation studies reveal residues critical for the activity of the mannitol permease from *Escherichia coli*

Erwin P.P. Vos, Ramon ter Horst, Bert Poolman, Jaap Broos *

Groningen Biomolecular Science and Biotechnology Institute, University of Groningen, Nijenborgh 4, 9747 AG Groningen, The Netherlands

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ABSTRACT

This paper presents domain complementation studies in the mannitol transporter, EII^{mtl}, from *Escherichia coli*. EII^{mtl} is responsible for the transport and concomitant phosphorylation of mannitol over the cytoplasmic membrane. By using tryptophan-less EII^{mtl} as a basis, each of the four phenylalanines located in the cytoplasmic loop between putative transmembrane helices II and III in the membrane-embedded C domain were replaced by tryptophan, yielding the mutants W97, W114, W126, and W133. Except for W97, these single-tryptophan mutants exhibited a high, wild-type-like, binding affinity for mannitol. Of the four mutants, only W114 showed a high mannitol phosphorylation activity. EII^{mtl} is functional as a dimer and the effect of these mutations on the oligomeric activity was investigated via heterodimer formation (C/C domain complementation studies). The low phosphorylation activities of W126 and W133 could be increased 7–28 fold by forming heterodimers with either the C domain of W97 (IIC^{mtl}W97) or the inactive EII^{mtl} mutant G196D. W126 and W133, on the other hand, did not complement each other. This study points towards a role of positions 97, 126 and 133 in the oligomeric activation of EII^{mtl}. The involvement of specific residue positions in the oligomeric functioning of a sugar-translocating EII protein has not been presented before.

1. Introduction

The phosphoenolpyruvate-dependent group-translocation system (PTS) is responsible for the transport of sugars and their concomitant phosphorylation in bacteria [1–3]. The mannitol-specific component of this system in *E. coli* is EII^{mtl}, and this protein consists of three domains, C, B, and A (Fig. 1). The C domain, the actual translocator, is embedded in the cytoplasmic membrane. The A and B domains are located at the cytoplasmic side of the membrane and carry out consecutive phosphoryl transfer reactions. Phosphoryl transfer from phosphoenolpyruvate (PEP) to mannitol proceeds via two cytoplasmic kinases Enzyme I (EI) and the histidine-containing protein (HPr) to His554 at the A domain, followed by transfer to Cys384 at the B domain. The B domain donates the phosphoryl group to mannitol while it is transported to the cytoplasmic side of the C domain (IIC^{mtl}).

EII^{mtl} is functional as a dimer both in membranes and in the detergent-solubilized state [4–8]; the crucial dimer contacts appear to be between the two C domains [9,10]. The formation of heterodimers has been used to demonstrate that EII^{mtl} is functional as dimer [10–14]. Mutants of EII^{mtl}, inactive by virtue of a mutation in the A, B, or C domain, could be reactivated by mixing with another mutant carrying the mutation in another domain [3]. For example, two EII^{mtl} mutants inactivated via mutating their phosphorylation sites at the A domain (H554A) or the B domain (C384S), respectively, formed an active heterodimer under *in vivo* and *in vitro* conditions (A/B domain complementation) [11,12]. Also A/C, and B/C domain complementation have been demonstrated for several EII members of the PTS system [3,15,16]. In this manuscript C/C domain complementation experiments are presented. If two inactive mutants form an active heterodimer, information is obtained about positions critical for the oligomeric activation of the enzyme. Four single-tryptophan (Trp)-containing mutants, containing a Trp at position 97, 114, 126 or 133, respectively, are used in the complementation experiments. These mutants were constructed to study the structure and dynamics of EII^{mtl} with Trp fluorescence and phosphorescence spectroscopy [17]. Residues 97, 114, 126 and 133 are located in a cytoplasmic loop (residues 70–134) between putative transmembrane helices II and III in the IIC^{mtl} topology model of Sugiyama et al. [18] (Fig. 2). Recent studies [17,19] suggest that residues in this loop are involved in the mannitol translocation process. Some of these residues are only accessible from the periplasmic side while others are only accessible from the cytoplasmic side [19]. Conformational changes in this part of IIC^{mtl} induced by mannitol binding or by EII^{mtl} phosphorylation have

Abbreviations: dPEG, decylpoly-(ethyleneglycol)300; PEP, phosphoenolpyruvate; Phe, phenylalanine; Trp, tryptophan; K_d, dissociation constant; PTS, phosphoenolpyruvate-dependent group-translocation system; EI, Enzyme I from the PTS system of *E. coli*; HPr, histidine-containing protein of *E. coli*

* Corresponding author. Tel.: +31 50 3634277; fax: +31 50 3634800.
E-mail address: j.broos@rug.nl (J. Broos).

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Nomenclature of the enzymes

EII^{mtl}, wild-type EII^{mtl} with Trps at position 30, 42, 109, and 117. G196D, wild-type EII^{mtl} containing an aspartic acid at position 196. Trp-less EII^{mtl}, EII^{mtl} in which the four native Trps are replaced by Phe. W97, W114, W126, and W133 are the single-Trp-containing EII^{mtl} mutants based on Trp-less EII^{mtl}. Wild-type IIC^{mtl}, C domain of the wild-type enzyme. Trp-less IIC^{mtl}, C domain of the Trp-less enzyme. IIC^{mtl}W97, IIC^{mtl}W114, IIC^{mtl}W126, and IIC^{mtl}W133 are the corresponding C domains of the single-Trp-containing EII^{mtl} mutants.

been reported [17,19]. Data presented in this work show that this part of IIC^{mtl} is also involved in the oligomeric activation of EII^{mtl}.

2. Materials and methods

2.1. Chemicals and reagents

Imidazole and L-histidine were from Fluka. Ni-NTA resin was from Qiagen inc. dPEG was synthesized by B. Kwant (Kwant High Vacuum Oil Recycling and Synthesis, Bedum, The Netherlands). D-[1-^{14}C]-mannitol (2.07 Gbq/mmol) was obtained from Amersham. D-[1-^{3}H(N)] mannitol (729 Gbq/mmol) was from NEN Research Products. EI and HPr were purified as described previously [20,21].

2.2. DNA techniques

2.2.1. Construction of pMaHismtlAP$_r$

To construct the plasmid pMaHismtlAP$_r$, bearing the *mtlA* gene which specifies EII^{mtl} with a thrombin cleavable N-terminal His-tag, the 84-mer PCR primer HismtlA 5′ - ACA TTA GGT ACC ATG CAT CAC CAT CAC CAT CAC AGC AGC GGC CTG GTG CCG CGC GGC TCG CAT ATG TCA TCC GAT ATT AAG ATC - 3′ was used in combination with a *mtlA* internal primer, that is 3′ - CCG ACG GCC GCA ATT G - 5′. The HismtlA primer contains a KpnI site necessary for cloning of the PCR fragment. The PCR product containing the 5′ part of the *mtlA* gene, including a sequence specifying the N-terminal His-tag, was digested with NcoI and KpnI and the fragment was subsequently ligated in pMamtlAP$_r$ [22]. pMamtlAP$_r$ has a KpnI site at the translation initiation codon of the *mtlA* gene that was introduced by the site-directed mutagenesis primer 5′ - GGA ACT GTA GGT ACC ACC CC - 3′ via the Kunkel method [23]. The new vector pMaHismtlAP$_r$ was then obtained by swapping the KpnI–NcoI fragment for the corresponding PCR product. Starting at the ribosome binding site, the 5′ *mtlA* flanking sequence in pMaHismtlAP$_r$ is 5′ - A AGG GGT GGT ACC ATG, which differs in four basepairs from the sequence 5′ - A AGG GGT GTT TTT ATG in pMamtlaP$_r$. The entire sequence was confirmed by nucleotide sequence analysis.

2.3. Construction of EII^{mtl} mutants

Site-specific mutagenesis of the *mtlA* gene generating single-Trp mutants W97 and W126 was performed according to Kunkel [23]. The mutations resulting in the single-Trp mutants W114 and W133 were introduced using the Quick Change Site-Directed Mutagenesis kit from Stratagene. Wild-type IIC^{mtl}, Trp-less IIC^{mtl}, IIC^{mtl}W97, IIC^{mtl}W114, IIC^{mtl}W126, and IIC^{mtl}W133 were created by the introduction of a stop codon at codon 338 in the wild-type EII^{mtl}, Trp-less EII^{mtl}, W97, W114, W126, and W133 genes, respectively. The primers were designed to create a new restriction site as well as the indicated amino acid substitution.

2.4. Growth of bacteria and preparation of membrane vesicles

Membrane vesicles were prepared from *E. coli* strain LGS322 [F$^-$ *thi-2*, *hisG1*, *argG6*, *metB1*, *tonA2*, *supE44*, *rpsL104*, *lacY1*, *galT6*, *gatR49*, *gatR50*, Δ(*mtlA'p*), *mtlD^c*, Δ(*gutR'MDBA-recA*)], carrying pMaHismtlAP$_r$. The LGS322 strain harbours a chromosomal deletion in the wild-type *mtlA* gene [24]. The procedures to grow LGS322/pMaHismtlAP$_r$ and to express EII^{mtl} were as described previously [22]. Membrane vesicles were prepared as described [25].

2.5. EII^{mtl} assays

Non-vectorial PEP-dependent phosphorylation of mannitol by EII^{mtl} in detergent solubilized membrane vesicles was measured as described [26]. The assay buffer contained 25 mM Tris–HCl, pH 7.6, 5 mM $MgCl_2$, 5 mM DTT, 5 mM PEP, 0.25% dPEG, 20 μM HPr, 0.33 μM EI, and rate–limiting amounts of EII^{mtl}. The samples were incubated for 10 min at 30 °C before the reaction was started with 60 μM [^{3}H]-mannitol. For complementation of the PEP-dependent phosphorylation of EII^{mtl} (mutants), saturating amounts of IIC^{mtl} or G196D were added to the assay buffer [13]. Then, the samples were incubated for 30 min at 30 °C to allow formation of heterodimers before the reaction was started with the addition of 60 μM [^{3}H]-mannitol. The very low activity of W126 and W126-IIC^{mtl}W126 was more difficult to measure, resulting in a relative large variation of 20–30% in the activity values. The EII^{mtl} concentration was determined by flow dialysis [27], thereby quantitating the number of mannitol binding sites. The assumption was made that one high affinity binding site is present per dimer [27].

For mannitol uptake experiments, LGS322 cells, expressing wild-type or mutant EII^{mtl}, were used. The cells were harvested at A_{600} of ~1, washed with 50 mM KP_i, pH 7.5, and resuspended to an A_{600} = 10 in the same buffer. The experiment was started by adding 10 μl 36 μM [^{14}C]-mannitol to 10 μl of cells in 80 μl 50 mM KP_i, pH 7.5, resulting in a final A_{600} of 1. The uptake was quenched by the addition of 2 ml of ice-cold 50 mM KP_i, pH 7.5, containing 1 mM $HgCl_2$ and the cell suspension was immediately filtered through Whatman GF/F. The filter was washed twice with 2 ml of the quenching solution. The

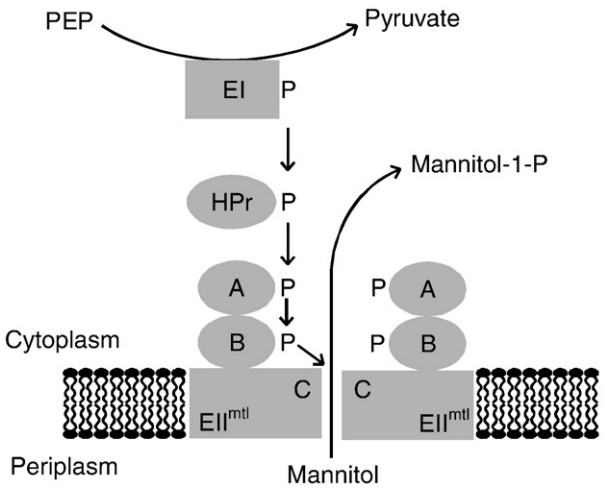

Fig. 1. Schematic representation of the mannitol-specific PTS of *E. coli*.

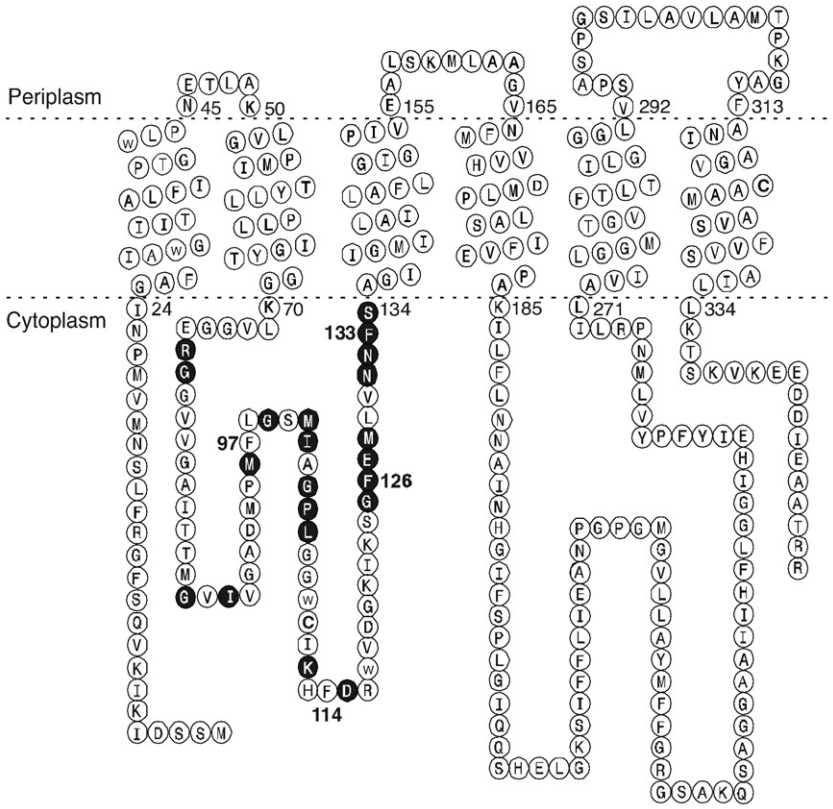

Fig. 2. Membrane topology model of the C domain of EII^{mtl} [18]. The conserved residues in the cytoplasmic loop between helices II and III in four EII^{mtl} proteins (EII^{mtl} and cryptic EII^{mtl} from *E. coli*, and EII^{mtl} from *S. carnosus* and *S. mutants*) are represented in black [2]. The positions where Trps were introduced are marked with numbers 97, 114, 126, and 133, respectively.

amount of radioactivity in the cells was quantified by liquid scintillation fluid counting.

3. Results

3.1. Engineering strategy

Mutations were introduced in the cytoplasmic loop between putative helices II and III in the IIC topology model of Sugiyama et al. [18] (Fig. 2) by replacing Phe at position 97, 114, 126, or 133 with Trp in Trp-less EII^{mtl}, yielding mutants W97, W114, W126, and W133, respectively. Like wild-type EII^{mtl}, Trp-less EII^{mtl}, W114, W126, and W133, show a high affinity binding for mannitol ($K_d \sim 100$ nM) [17,27]. Thus Phe to Trp mutations in Trp-less EII^{mtl} at position 114, 126, and 133 did not affect the K_d for mannitol binding as determined in membrane vesicles solubilized with the detergent dPEG. Moreover, wild-type IIC^{mtl}, that is EII^{mtl} without the A and B domains, Trp-less IIC^{mtl}, and the single-Trp IIC^{mtl} mutants of W114, W126 and W133 retained the high affinity for mannitol (data not shown). Only in the case of W97 and IIC^{mtl}W97 the binding affinity was much lower ($K_d \sim 2$ μM) [17].

The fact that three out of four mutants were nearly inactive as homodimers (see below at 3.3) prompted us to study these mutants in more detail in C/C domain complementation experiments with each other and with G196D, an EII^{mtl} mutant completely inactivated by a single point mutation in the C domain at position 196 [13].

3.2. Fermentation and mannitol uptake activities of wild-type and mutant EII^{mtl}

As a first qualitative measure of transport activity of wild-type and mutant EII^{mtl}, the corresponding genes were expressed in the *E. coli* LGS322 strain containing a chromosomal deletion in the wild-type *mtlA* gene, encoding EII^{mtl} [24]. Fermentation was assayed on MacConkey-mannitol indicator plates. Expression of wild-type EII^{mtl}, Trp-less EII^{mtl}, W114, and W133 resulted in red colonies, whereas W97 and W126 yielded pink colonies (Table 1). This indicates that W97 and W126 are severely defective in mannitol transport. The decrease in transport activity of these mutants was confirmed in uptake assays, wherein W97 proved to be nearly inactive and W126 displayed 33% of the wild-type EII^{mtl} transport activity (Table 1). Remarkably, mutant W114 shows an uptake activity 22% higher than wild-type EII^{mtl}.

3.3. Mannitol phosphorylation activity

The mannitol phosphorylation activity of EII^{mtl} is conveniently assayed both in intact membranes and in the detergent-solubilized

Table 1
Fermentation and mannitol uptake activities of wild-type and mutant EII^{mtl}

Enzyme species	Phenotype[a]	Rate of mannitol uptake[b] (nM/min)	Relative mannitol uptake activity (in %) compared to wild-type EII^{mtl}
Wild-type EII^{mtl}	Red	2760±410	100
Trp-less EII^{mtl}	Red	2700±320	98
W97	Pink	220±20	8
W114	Red	3380±340	122
W126	Pink	900±70	33
W133	Red	2040±240	74

[a] A red phenotype indicates transport and fermentation of mannitol; a pink phenotype indicates low levels of transport and/or fermentation. The fermentation studies were performed with 55 mM mannitol in the plate.
[b] The rate of mannitol uptake was determined at a [^{14}C]-mannitol concentration of 3.6 μM.

Table 2
Non-vectorial mannitol phosphorylation activities of EII^{mtl} and EII^{mtl}/IIC^{mtl} heterodimers

Enzyme species	Rate of phosphorylation (min^{-1})		
	Homodimer	Complementing protein	
		Allelic IIC^{mtl}	Wild-type IIC^{mtl}
Wild-type EII^{mtl}	2780±50	5000±260	5000±260
Trp-less EII^{mtl}	2060±210	3920±180	3710±410
W97	110±10	190±10	2180±270
W114	1520±220	2130±170	2590±380
W126	60±20	70±20	2070±180
W133	160±60	240±40	1110±130
Cartoon representation of the assayed EII^{mtl} species			

state by determining the rate of phosphorylation of radioactive mannitol. The phosphorylation activity of Trp-less EII^{mtl} is high and only 25% lower than wild-type EII^{mtl} (Table 2, first column). A Phe to Trp mutation at position 114 in the Trp-less EII^{mtl} background did not severely affect the phosphorylation activity. In contrast, the phosphorylation activity of W97, W126, and W133 was less than 10% of the Trp-less EII^{mtl} activity (Table 2).

3.4. Oligomeric state of EII^{mtl}

Only dimeric EII^{mtl} exhibits Mtl phosphorylation activity [28]. Boer et al. [13] studied the activity of heterodimeric EII^{mtl} consisting of a wild-type EII^{mtl} subunit (A, B and C domains) and a wild-type IIC^{mtl} subunit [10] (Fig. 3). In these experiments, wild-type EII^{mtl} was mixed with excess (inactive) wild-type IIC^{mtl} in buffer containing dPEG detergent. Under these conditions heterodimers are formed [14]. After complete exchange, each EII^{mtl} subunit is complexed with a IIC^{mtl} subunit, yielding a heterodimer concentration twice as high as the homodimeric EII^{mtl} concentration. When the phosphorylation activity was measured of this sample a doubling of the activity was observed compared to the activity before excess wild-type IIC^{mtl} was introduced (Fig. 3). From this work it was concluded that the specific activity of the wild-type EII^{mtl} homodimer is comparable to the specific activity of the wild-type EII^{mtl}/wild-type IIC^{mtl} heterodimer, implying that only one A- and one B domain are essential per two IIC^{mtl} domains [10]. Since the contacts between the subunits in the dimer are via the C domain, the mutations introduced in W97, W114, W126, and W133 could well result in a significant fraction of the monomeric form. When a fraction of these solubilized mutant enzymes is present as a monomer, mixing with excess IIC^{mtl} domain should result in a more than doubling of the phosphorylation activity because of heterodimer formation induced by mass action.

First, we addressed the question whether or not the EII^{mtl} mutants can form functional dimers. Complementation experiments [13] were

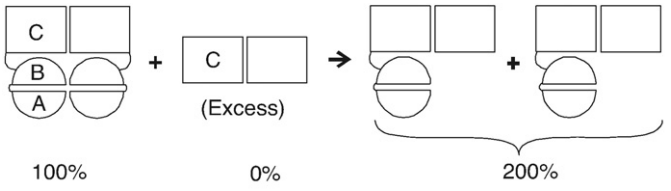

Fig. 3. Schematic representation of the C/C domain complementation assay of wild-type EII^{mtl} with wild-type IIC^{mtl}: Mixing of EII^{mtl} with excess IIC^{mtl} in buffer containing dPEG detergent results in heterodimer formation. After equilibration, the concentration of heterodimers becomes twice the used EII^{mtl} concentration. The mannitol phosphorylation activity of the EII^{mtl} sample (100%) becomes 200% after mixing with excess IIC^{mtl} (inactive). From this, it was concluded that EII^{mtl} and the formed heterodimer exhibit the same specific mannitol phosphorylation activity [10].

Table 3
Non-vectorial mannitol phosphorylation activities of single-Trp EII^{mtl} mutants and their heterodimers

Enzyme species	Rate of phosphorylation (min^{-1})				
	Homodimer	Complementing protein			
		EII^{mtl} G196D	IIC^{mtl} W97	IIC^{mtl} W126	IIC^{mtl} W133
W97	110±10	190±10	190±5	410±40	440±30
W126	60±20	1770±100	1760±120	70±20	140±50
W133	160±60	1360±20	1250±150	200±60	240±40

set up by mixing the Trp-less enzyme and each of the single-Trp mutants with an excess of its allelic IIC^{mtl}, that is the same mutant now truncated with a stop codon at residue 338 in the C/B domain linker. In this manner, it is possible to address the effect of the specific mutation on the monomer–dimer equilibrium (Fig. 3). The results of these experiments are presented in Table 2 (column 2). As a control, wild-type EII^{mtl} and excess wild-type IIC^{mtl} gave the expected doubling (~180%) in phosphorylation activity (Table 2). The phosphorylation activities of Trp-less EII^{mtl} and the single-Trp mutants, increased 140–190% upon addition of an excess of allelic IIC^{mtl} (120% for W126). These results indicate that Trp-less EII^{mtl} and the single-Trp mutants are at least largely in the dimeric state under these conditions.

3.5. Complementation of W97, W126, W133 with wild-type IIC^{mtl}

In a second series of complementation experiments, we addressed the question whether the low activities of the W97, W126, and W133 mutants are the result of the pair wise presence of the mutation in the homodimer or that a single mutation per dimer caused the defect. To investigate this possibility, the mutants were complemented with wild-type IIC^{mtl}. Complementation of solubilized W97, W126, and W133 with excess solubilized wild-type IIC^{mtl} domain dramatically increased the specific activity (Table 2, last column). The resulting phosphorylation activities of the W97, W126, and W133 heterodimers with wild-type IIC^{mtl} ranged from 30 to 70% of the activity of the Trp-less EII^{mtl}/Trp-less IIC^{mtl} heterodimer. The high phosphorylation activities of these heterodimers indeed suggest that the pair wise presence of the mutation in the homodimer is causing the very low phosphorylation activities of W97, W126 and W133. To rule out the possibility that the presence of the wild-type IIC^{mtl} subunit is responsible for the high phosphorylation activities of the heterodimers, C/C complementation experiments were also performed with inactive EII^{mtl} mutants.

3.6. Complementation of W97, W126, and W133 with inactive mutants

Complementation of the solubilized W97, W126, and W133 mutants with each other are presented in Table 3. Although the activities of W97, W126, and W133 are low (Table 2), they cannot be used in excess in the complementation experiment because the phosphorylation activity generated by using excess W97, W126, or W133 exceeds the phosphorylation activity of the heterodimers. Therefore, the IIC^{mtl} mutants were used in these experiments. These mutants have properties similar to the wild-type- and Trp-less IIC^{mtl} with respect to heterodimer formation (Table 2). G196D, a mannitol binding and phosphorylation defective EII^{mtl} mutant was included in this set of experiments [13]. The absence of activity of G196D allowed the use of full length enzyme II. Results of these complementation experiments are presented in Table 3 and can be summarized as follows:

- Complementation of W126 or W133 with an excess of IIC^{mtl}W97 or G196D resulted in active heterodimers with an activity 8- to 30-fold higher than the activity of W126 or W133 homodimers.

- Complementation of W126 with excess IIC^{mtl}W133, or W133 with excess IIC^{mtl}W126, did not result in a significant difference in phosphorylation activity as observed if W126 and W133 were mixed with an excess of their allelic IIC^{mtl} domains (Table 2). Thus, W126 and W133 were not able to suppress each other's defect.
- Complementation of W97 with IIC^{mtl}W126 or with IIC^{mtl}W133 resulted in an activity only 4-fold higher than that found for W97. In contrast W97 forms active heterodimers with wild-type IIC^{mtl} (Table 2, last column). Complementation of W126 and W133 with IIC^{mtl}W97 also results in active heterodimers (Table 3). One explanation for the low activity of W97-IIC^{mtl}W133 and W97-IIC^{mtl}W126 could be a further lowering of the mannitol binding affinity of W97 (K_d ~ 2 μM) when complexed with one of these two IIC^{mtl} domains. The phosphoryl group at Cys384 of W97 then needs to be transferred to the C domain of IIC^{mtl}W126 or IIC^{mtl}W133. Only these IIC domains show a high affinity for mannitol. This inter-subunit phosphoryl transfer is expected to result in a significantly lower phosphorylation activity compared to the intra-subunit phosphoryl transfer activity of the W126/IIC^{mtl}W97 and W133/IIC^{mtl}W97 heterodimers. This is in line with earlier experiments with G196D/IIC^{mtl} heterodimers [13].
- IIC^{mtl}W97 and G196D, both unable to bind mannitol with high affinity, did not complement each other (data not shown).

Taken together, the W126 and W133 mutants, both showing high affinity mannitol binding and a low phosphorylation activity, could complement W97 and G196D, both lacking high affinity mannitol binding and mannitol phosphorylation activity. However, the combinations W126 with IIC^{mtl}W133, W133 with IIC^{mtl}W126 or W97 with G196D did not complement each other.

4. Discussion

The mannitol phosphorylation activity of four single Trp mutants of EII^{mtl} with a Trp in the first putative cytoplasmic loop of the IIC^{mtl} (residues 70–134, Fig. 2) is presented. Likely, these residues form an ordered part of the C domain, as the spectroscopic properties of the four single-Trp mutants correspond with Trp positions in a structured microenvironment [17]. In recent topology models this part of IIC^{mtl} is presented forming two short helices protruding into the bilayer [19] or forming a transmembrane helix and a periplasmic loop [29]. Sequence alignments between EII^{mtl} proteins from different species revealed that this cytoplasmic loop is well-conserved (residues 96 to 106 and 125 to 134) [2] (Fig. 2). Residues F126 and F133 are conserved in the 4 EII^{mtl} proteins used in this alignment as are the neighbouring residues M96, and D115 of F97 and F114, respectively. Phe to Trp substitutions resulted in the loss of high affinity mannitol binding (W97), a much lower phosphorylation activity (W97, W126, and W133), and no (W97) or lower mannitol transport activity (W126). The mannitol binding-, transport-, and phosphorylation activities of W114 were comparable to those of the Trp-less enzyme. Except for position 114, the mutations introduced in this loop are within the two conserved regions. Several studies have demonstrated that phosphorylation of mannitol requires dimeric EII^{mtl} but no structural information about the activity-linked oligomeric interaction for EII^{mtl} or other members of the EII family is available. In the 7 Å 2D projection map of (homo)dimeric IIC^{mtl} on which no symmetry was imposed, differences in electron densities between the two subunits is visible [30]. Thus, two, in-sequence, identical subunits induce structural differences on each other upon dimerization and these differences might be related to the oligomeric activation of EII^{mtl}.

Second-site suppressor studies are powerful means to identify neighboring residues in a protein. The presented C/C domain complementation approach yields similar information, but now between two subunits forming a functional dimer. Inactive homodimers with normal heterodimer formation properties are needed for this approach. The complementation of W97, W126, and W133 with excess allelic IIC domain resulted in a nearly doubling of the specific activities (120% for W126), a result indicating that W97, W126, and W133 are in dimeric form under the experimental conditions. Apparently, these three mutations do not affect EII^{mtl} dimerization. The low mannitol phosphorylation activity of W97 could be due to the poor affinity of this mutant for mannitol; this aspect was not investigated further.

The non-vectorial phosphorylation activities of the investigated homodimeric EII^{mtl} mutants are lower as found for wild-type EII^{mtl} (Table 2, column 1). In contrast, the mannitol uptake activity of Trp-less EII^{mtl} was essentially similar to wild-type while the uptake activity of W114 was 122% the activity of wild-type (Table 1). W133 shows a good uptake activity but a very low non-vectorial phosphorylation activity. These results show that the four W → F mutations introduced in Trp-less protein and the extra F → W mutation at position 114 and 133 in W114 and W133, respectively, affect in particular the non-vectorial phosphorylation activity not the mannitol uptake activity.

W126 and W133 show low phosphorylation activities as homodimers or when combined with each other in a heterodimer. Thus, the presence of a two Trp instead of two Phe residues in both monomers at both 126 positions, at both 133 positions or at one 126 and one 133 position in the dimer causes a defect in mannitol phosphorylation. The individual W126 and W133 monomers can be complemented with IIC^{mtl}W97 and G196D, mutants without any phosphorylation activity and unable to bind mannitol with high affinity. Our results reveal a role of residue positions 97, 126 and 133 in the oligomeric activation of the enzyme II-mannitol transporter. Interestingly, the spectroscopic characterization of these mutants shows that the microenvironment at position 97, 126 and 133 changes significantly when mannitol is bound; for 97 and 126 a loosening of the microenvironment was observed, while the protein becomes more structured around position 133 [17]. For position 114, no clear structural changes were noted upon mannitol binding. The large impact of residues 126 and 133 on the phosphorylation activity rather than the mannitol binding affinity suggests that they are involved in the phosphoryl transfer from Cys384 in the B domain to mannitol bound at the C domain. Chemical cross-linking of a Cys at position 124 (C domain) and the active-site Cys (position 384) in the B domain provided structural information that this region is indeed at the B/C domain interface [31]. Mutants in which a Cys was introduced at position 85, 90, 91, or 100, respectively, using a single Cys EII^{mtl} construct as basis (Cys at 384), showed no or reduced mannitol binding affinities and mannitol phosphorylation activities. Taken together, this and previous studies show that this part of IIC^{mtl} contains residues important for EII^{mtl} activity.

Residue positions in the C domain, critical for the activity of the *E. coli* glucose transporter (EII^{glc}) have been reported [16,32,33]. In EII^{glc}, residues 79–151 correspond to 70–134 in EII^{mtl} [2]. Mutants G149S and K150E of EII^{glc} showed strongly reduced transport activities, together with good glucose phosphorylation activity. It was concluded that residues 149 and 150 are directly involved in sugar binding/translocation [32].

Further work is needed to elucidate in more detail the role of this part of IIC^{mtl} in the mannitol transport mechanism. Whether or not it harbors the mannitol binding site is currently investigated with resonance energy transfer experiments using the four single-Trp mutants and the chromophoric mannitol analog azi-mannitol [34].

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