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

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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*

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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

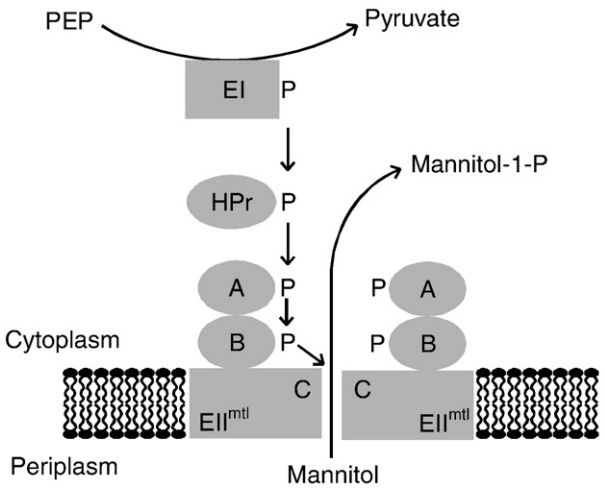

Fig. 1. Schematic representation of the mannitol-specific PTS of *E. coli*.