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Technical Note

The V-motifs facilitate the substrate capturing step of the PTS elevator mechanism

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ABSTRACT

We propose that the alternative crystal forms of outward open UlaA (which are experimental, not simulated, and contain the substrate in the cavity) can be used to interpret/validate the MD results from MalT (the substrate capture step, which involves the mobile second TMSs of the V-motifs, TMSs 2 and 7). Since the crystal contacts are the same between the two alternative crystal forms of outward open UlaA, the striking biological differences noted, including rearranged hydrogen bonds and salt bridge coordination, are not attributable to crystal packing differences. Using transport assays, we identified G58 and G286 as essential for normal vitamin C transport, but the comparison of alternative crystal forms revealed that these residues to unhinge TMS movements from substrate-binding side chains, rendering the mid-TMS regions of homologous TMSs 2 and 7 relatively immobile. While the TMS that is involved in substrate binding in MalT is part of the homologous bundle that holds the two separate halves of the transport assembly (two proteins) together, an unequal effect of the two knockouts was observed for UlaA where both V-motifs are free from such dimer interface interactions.

1. Introduction

Seldom is the same conformational state of a protein crystallized twice in alternative crystal forms, using the same crystallization conditions, in the same lattice, but in two different space groups (UlaA in the outward open state; $P2_1A$ which is more unusual, and C2A which is more frequent). In this article, we propose that this scenario presents valuable information about structural flexibility, similar to the information obtained from temperature factors or from Normal Mode Analysis, yet coming from a different and independent information source. We attempt to subtract the estimates of local instability from the different information sources, to gauge where they agree and disagree. To aid the comparison, we establish a reference point in UlaA by a functional transport assay and confirm it by a superimposition experiment. These reference points are two local and homologous regions of local stability, G58 and G286, and assist in the interpretation of comparative instability data.

What originally prompted our analysis was the fact that two glycines 58 and 286 that cropped up in a functional analysis of UlaA did not appear to be in mobile regions of the UlaA protein based on temperature factor data. This is in contrast to the notion that other well known mid-TMS glycines provide flexibility, such as the mid-TMS glycines of spiny helices of APC transporters (including Gly 25 and 206 of AdiC, PDB: 3L1L) (Västermark and Saier, 2014). While Gly25 and 206 apparently interrupt the helical structure of the substrate channel proximal TMSs, they do not revent measurable flexibility in temperature factor plots. Glycines 58 and 286 of UlaA are, in contrast to such flexible glycines of APC transporters, located near the membrane and not in the protein interior or substrate translocation channel. We wanted to determine using NMA if the apparent lack of flexibility could be an artifact caused by the crystal packing environment and discovered that unlike many proteins, UlaA has two alternative crystal forms in the same conformational state, providing a third source of flexibility information.

Abbreviations: C2A, $P2_1A$, space group names; APC, Amino acid-Polyamine organoCation superfamily of secondary carriers; PTS, bacterial Phospho-Transferase System; NMA, Normal Mode Analysis.

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2. Purpose

The rationale for this research is provided by considerations concerning the so called "Elevator Mechanism" (Vastermark and Saier, 2016). One of the four steps of the "Elevator Mechanism" involves a transition in which the substrate is captured (outward open to outward occluded transition). Recently, the outward occluded conformation of MalT of the GFL superfamily was solved, but the "substrate capturing step" could only be simulated using MD (McCoy et al., 2016). We have proposed that secondary structural elements can be mapped between the GFL superfamily (MalT, ChbC) and the ascorbate-galactitol (AG) superfamily (UlaA) (Vastermark and Saier, 2016) (Fig. 1, Table 1 in Vastermark et al. (2016) or Fig. S1 and Table S1). For this reason, we believe that the MD simulation of the substrate capturing step of GFL superfamily transporters can be partially "validated" by studying the alternative crystal forms of UlaA. Because the crystal contacts are the same between the alternative crystal forms, the differences seen cannot be attributed to contacts. Instead, we believe the alternative crystal forms can be found in the intermediates of the MD simulation (McCoy et al., 2016). The MD simulation suggests that it is the second half of the V-motifs (TMSs 2 and 7) that are involved in capturing the substrate, and that it is the mobile nature (presumably mediated by the critical glycines) that allows capture of the substrate (cf. outward open to occluded transition in UlaA, Fig. S2). In the alternative crystal forms of the outward open UlaA, the substrate is present, but in the transition to the outward occluded state, it is found that many salt bridges and substrate coordinating hydrogen bonds are rearranged. An interesting parallel can be drawn to the MFS (Radestock and Forrest, 2011) where a conserved movement of TMS11 releases the substrate on the inward side.

3. Background: PTS protein structures

The phosphoenolpyruvate (PEP):sugar phosphotransferase system (PTS) family of group translocators (TC #4.A; see TCDB, tcdb.org) is conceptually different from other major types of transport systems (e.g., primary active, secondary active or channel-based), in that the substrate is phosphorylated during transport (Vastermark and Saier, 2014). The transmembrane domains of PTS porters are traditionally designated Enzymes IIC (IICs), and can be grouped into several superfamilies. Members of different superfamilies are believed to be non-homologous (Saier et al., 2005). Other constituents of the PTS include the energy-coupling proteins, Enzyme I and HPr, and the phosphoryl transfer proteins, Enzymes IIA and IIB (IIA and IIB). High resolution structural information has been available for some time for two IIC proteins: the vitamin C transporter UlaA from *E. coli*, and the diacetylchitobiose transporter ChbC from *Bacillus cereus*.

4. Background: UlaA (TC# 4.A.7.1.1)

Bacteria, including intestinal *E. coli*, ferment vitamin C (L-ascorbic acid) as a carbon source, but mammals use it as a cytoplasmic reducing agent (Levine et al., 2011). Vitamin C is imported into *E. coli* by the transport system Ula (Utilization of ascorbic acid), which includes three proteins, IIA, IIB, and IIC^{Ula} (Luo et al., 2015; Zhang et al., 2003; Hvorup et al., 2003). In contrast, vitamin C uptake across the mammalian intestinal brush border is mediated by a sodium-dependent secondary carrier. The *E. coli* transporter has 10–25 times higher affinity (lower K_m) than intestinal secondary carriers (Jeckelmann et al., 2014; Boggavarapu et al., 2013), indicating that the bacteria can effectively compete with the intestinal mucosal cells.

The resolution of the X-ray structures of UlaA is 1.65 and 2.35 Å for the C2 and $P2_1$ space groups, respectively. The symmetry-related core domain coordinates vitamin C binding. Two major types of PTS group translocators include the "AG" type of which UlaA is a member (The PTS L-Ascorbate (L-Asc) Family (Zhang et al., 2003)), and the lactose family of the Glc-Fru-Lac ("GFL") superfamily (Nguyen et al., 2006), of which the chitobiose transporter ChbC is a member (McCoy et al., 2015; Cao et al., 2011). The AG and GFL superfamilies are not believed to be homologous and may have different evolutionary origins (Saier et al., 2014). In fact, at least four independently evolving EIICs are currently believed to exist within the PTS domain (Saier et al., 2005).

Since static light scattering experiments suggested that UlaA is a dimer in solution, the homodimeric partner in the C2 space group (PDB: 4RP9), indicated as C2A′, could be generated by a crystallographic two-fold axis (Luo et al., 2015). C2A and C2A′ are symmetry-related; therefore both of them have the same conformation. Only in the $P2_1$ space group (PDB: 4RP8) do the two different conformations, $P2_1$A and $P2_1$B, exist. From the structural superimposition, C2A is equivalent to (=) C2A′ = $P2_1$A ! = $P2_1$B (C2A, C2A′, and $P2_1$A are outward states, whereas $P2_1$B is the "occluded" state). In fact, we want to refute a suggestion (Luo et al., 2015) that the two protomers in the UlaA dimer function independently, as this suggestion disagrees with the cooperative model proposed for ChbC (Luo et al., 2015). The superimposition of C2A′ and $P2_1$B showed that while the structures are very similar, the core domain rotates 4.33° compared to the V motif, causing some atoms to move as much as 7 Å (Luo et al., 2015). The "rigid body" model is partly based on the fact that no conformational changes occur within the core domains, although rotational movement can be measured.

5. Methods

5.1. Protein expression and purification

We selected several homologs of UlaA from different prokaryotic genomes. Their cDNAs were cloned into pET15b and pET21b (Novagen). Please refer to published protocol in Luo et al. (2015) for the procedures used.

5.2. Preparation of proteoliposomes

Proteoliposomes were prepared following published protocols (Fang et al., 2007; Reig et al., 2007). An *E. coli* polar lipid extract (Avanti Polar Lipids) was prepared in CM buffer [chloroform: methanol, 3:1 (vol/vol)] to a final concentration of 50 mg/mL and then dried under a stream of nitrogen to remove the solvent and obtain thin layer dry lipids in a glass tube. The dried lipids were resuspended in inner reaction buffer (containing 25 mM Tris-HCl, pH 8.0, 150 mM NaCl) by vortexing for 20 min to yield a final lipid concentration of 20 mg/mL. After 10 cycles of quick freezing and thawing, the liposomes were extruded at least 21 times in an Avestin extruder through a 400-nm polycarbonate filter (Avanti) to obtain unilamellar vesicles of a homogeneous size. Protein concentration was determined using Bio-Rad reagents (Bradford Assay), and the liposomes were mixed with purified protein at a concentration of 50 μg/mg lipids. The ratio (by weight) of protein to lipid used to make proteoliposomes was 0.05. To destabilize the liposomes, β-D-octyl glucoside (OG) was added to a final concentration of 1.2%, followed by incubation at 4 °C for 2 h. DDM and OG were removed by incubating with 300 mg/mL Bio-Beads (Bio-Rad) overnight and then with 100 mg/mL Bio-Beads for 2 h. After five cycles of quick freezing and thawing, the proteoliposomes were extruded 21 times in the extruder through a 400-nm polycarbonate filter.

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