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Crystallization Notes

2D and 3D crystallization of a bacterial homologue of human vitamin C membrane transport proteins

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ABSTRACT

Most organisms are able to synthesize vitamin C whereas humans are not. In order to contribute to the elucidation of the molecular working mechanism of vitamin C transport through biological membranes, we cloned, overexpressed, purified, functionally characterized, and 2D- and 3D-crystallized a bacterial protein (UraDp) with 29% of amino acid sequence identity to the human sodium-dependent vitamin C transporter 1 (SVCT1). Ligand-binding experiments by scintillation proximity assay revealed that uracil is a substrate preferably bound to UraDp. For structural analysis, we report on the production of tubular 2D crystals and present a first projection structure of UraDp from negatively stained tubes. On the other hand the successful growth of UraDp 3D crystals and their crystallographic analysis is described. These 3D crystals, which diffract X-rays to 4.2 Å resolution, pave the way towards the high-resolution crystal structure of a bacterial homologue with high amino acid sequence identity to human SVCT1.

1. Introduction

Vitamin C (L-ascorbic acid, ascorbate) belongs to the water-soluble vitamins. It is involved in numerous enzymatic reactions and acts as an antioxidative agent preventing oxidative stress in cells and tissues by scavenging free radicals. In contrast to most organisms, humans and higher primates are unable to convert glucose to L-ascorbic acid and are therefore dependent on dietary vitamin C uptake (May, 2011). In humans, ascorbate enters the cell through the sodium-dependent vitamin C transporters SVCT1 (SLC23A1) and SVCT2 (SLC23A2). These transporters are members of the SLC23 (solute carrier 23) gene family (Burzle et al., 2013) and, according to the Transporter Classification Database (http://www.tcdb.org/), belong to the nucleobase/cation symporter 2 (NCS2) family also known as the nucleobase/acorbate transporter (NAT) family. The NAT family includes more than 2000 putative members derived from all major taxa of organisms, but so far, only a few members were characterized (Frillingos, 2012). Only recently, we reported the first low-resolution structure of human SVCT1 showing that this human transport protein exists as monomer and dimer in the plasma membrane and also when solubilized in detergent (Boggavarapu et al., 2013). The monomeric state is in-line with the crystal structure of the uracil/H^+ symporter (UraA; UniProtKB: P0AGM7; PDB: 3QE7) from *Escherichia coli* (*E. coli*) (Lu et al., 2011), which has sequence identities below 20% to human SVCTs. The crystal structure of UraA was solved to a resolution of 2.8 Å and displays the first and so far the only high-resolution structure of a NAT-family member (Lu et al., 2011). This structure revealed a new fold consisting of 14 transmembrane domains, which divide into two inverted repeats. Furthermore, it was shown that the NAT-signature sequence motif is located within the membrane-spanning region (Lu et al., 2011) and not as previously predicted in a cytoplasmic loop (Burzle et al., 2013). Using this structure, important functional residues could be verified by cross-checking a library containing 220 Cys-scanning mutants of the Xanthine permease (XanQ; UniProtKB: P67444) from *E. coli* with a homology model based on the UraA crystal structure (Karena and Frillingos, 2011). However, structural information on NAT-family members and in particular on human SVCTs remains scarce. In order to understand the transport mechanism of these important transporters, additional structural information is indispensable, e.g. structures of the transporter at high-resolution in different conformational states of the transport cycle. Structure elucidation of eukaryotic membrane proteins remains notoriously difficult. Although tremendous efforts in heterologous overexpression were reported lately, it remains a great challenge to

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overexpress human membrane proteins at a quality and expression level suitable for 3D crystallization and structure solution (Chaudhary et al., 2012). Moreover, eukaryotic post-translational protein modifications, e.g. glycosylation, may impair the growth of 2D and in particular 3D crystals. Therefore, structure elucidation of bacterial homologues, which are relatively easy to overexpress and not glycosylated, still remains a valuable tool to unravel the architecture and mode of action of transporters and membrane proteins in general.

In this crystallization note we describe the biochemical and functional characterization as well as the 2D and 3D crystallization of a bacterial NAT-member, the uracil permease from *Dethiosulfovibrio peptidovorans* (UraDp, UniProtKB: D2Z8A6), which has 29% amino acid sequence identity to human SVCT1. UraDp was purified to high purity and homogeneity, and the binding specificity and dissociation constant (K_D) of uracil was determined by scintillation proximity assay (SPA) (Harder and Fotiadis, 2012). Negative stain transmission electron microscopy (TEM) of purified UraDp indicated a monomeric state of the protein when solubilized in detergent. 2D crystallization of purified UraDp yielded tubular crystals and allowed the calculation of a projection structure by negative stain TEM. Importantly, 3D crystals of UraDp could be successfully grown and a full X-ray diffraction data set at 4.2 Å resolution recorded.

2. Results and discussion

UraDp was overexpressed in *E. coli* and extracted from isolated membranes by solubilization in the detergent n-nonyl-*β*-D-glucopyranoside (NG; Fig. 1A, lane 1). Nickel affinity chromatography followed by proteolytic digestion yielded ~650 μg of pure UraDp protein per liter of bacterial cell culture (Fig. 1A, lane 2). The purified protein was highly homogeneous as judged by size-exclusion chromatography (SEC) (Fig. 1B) and single particle analysis by negative stain TEM (Fig. 2A). Top view particles showed an elliptical shape of UraDp with a central stain filled cavity (Fig. 2B). The dimensions of these particles are ~8 nm × ~7 nm and similar to top views of monomeric transport proteins of similar molecular weight (Casagrande et al., 2009; Reig et al., 2007; Weitz et al., 2007). These results point to a monomeric state of UraDp when purified in NG.

In the UniProtKB the UraDp membrane protein is annotated as a xanthine/uracil/vitamin C permease. This annotation is supported by the presence of a Glu-residue at the 2nd position within the NAT-signature sequence motif, which predicts UraDp to be a uracil permease (Frillingos, 2012). To verify the hypothetical substrate specificities mentioned above (i.e., xanthine/uracil/vitamin C) experimentally, we performed the SPA using purified UraDp and [^{3}H]uracil (Fig. 3A). Because of the superior stability of UraDp in the relatively mild detergent n-dodecyl-β-D-maltopyranoside (DDM) (Prive, 2007), SPA experiments were performed with protein purified in DDM. SPA clearly demonstrated that uracil binds to UraDp. Furthermore, SPA also indicated that vitamin C decreased [^{3}H]uracil binding by ~40%. In contrast, there was virtually no effect if xanthine was used as competitor to uracil (Fig. 3A). Uracil binds to the purified UraDp protein with a K_D of 190 ± 10 nM (Fig. 3B) indicating that it is a high-affinity transporter. This K_D is twofold stronger than the K_D obtained for UraA (410 ± 70 nM, (Lu et al., 2011)).

2D crystals of purified UraDp were successfully grown using the dialysis method (Jap et al., 1992; Kuhlbrandt, 1992). Tubular-shaped crystals spanned over several micrometers whereas the width was rather small with an average diameter of ~100 nm (Fig. 4A and B). Electron micrographs of straight regions of well-ordered crystals were used to calculate a projection structure of

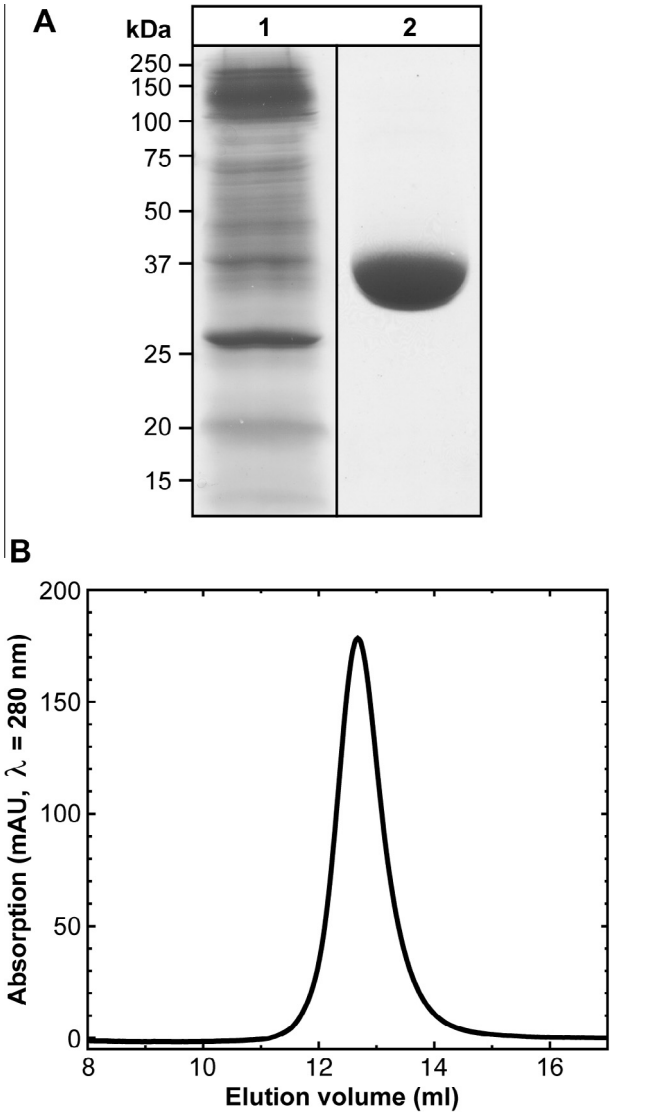

Fig.1. SDS–PAGE and analytical SEC of UraDp purified in NG. (A) SDS–PAGE of a representative UraDp purification. Lane 1 shows the NG-solubilized membrane fraction prior nickel affinity chromatography and lane 2 the purified and HRV 3C cleaved UraDp protein (~20 μg) on 13.5% SDS/polyacrylamide gels. (B) Analytical SEC of affinity chromatography purified and HRV 3C cleaved UraDp protein eluted with 20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 2% glycerol, 0.4% NG.

negatively stained UraDp at ~18 Å resolution (Fig. 4C and Supplementary Table 1). The symmetry of 2D UraDp crystals was assigned to *p1* and the unit cell determined to be a = 72 Å, b = 70 Å, γ = 100°. In order to estimate the number of UraDp proteins harbored per unit cell, the unit cell area was determined and compared to areas per monomer derived from 2D crystals of membrane proteins of similar monomeric molecular weight as UraDp (49 kDa), e.g. the L-arginine/agmatine antiporter AdiC (49 kDa; (Casagrande et al., 2008)), the peptide transporter DtpD (55 kDa; (Casagrande et al., 2009)), the membrane domain of the glucose transporter IIC^{glc} (43 kDa; (Jeckelmann et al., 2011)) and the oxalate transporter OxalT (44 kDa; (Heymann et al., 2001)). The area per monomer of these reference 2D protein crystals was calculated to be in the range of 2000–2800 Å^2. Because the unit cell area of UraDp is ~5000 Å^2, we assume the presence of two UraDp monomers per unit cell. This represents a non-crystallographic dimer since the oligomeric state of the detergent-solubilized UraDp was found to be monomeric as shown by single particle analysis (Fig. 2).