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Residues of a proposed gate region in type I ATP-binding cassette import systems are crucial for function as revealed by mutational analysis

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ABSTRACT

The type I ATP-binding cassette (ABC) importer for positively charged amino acids of the thermophilic bacterium *Geobacillus stearothermophilus* consists of the extracellular solute binding protein, ArtJ, and a homodimer each of the transmembrane subunit, ArtM, and the nucleotide-binding and -hydrolyzing subunit, ArtP. We have investigated the functional consequences of mutations affecting conserved residues from two peptide regions in ArtM, recently proposed to form a 'gate' by which access of a substrate to the translocation path is controlled (Hollenstein et al., 2007 [14]). Transporter variants were reconstituted into proteoliposomes and assayed for ArtJ/arginine-stimulated ATPase activity. Replacement of residues from region 1 (Arg-63, Pro-66) caused no or only moderate reduction in ATPase activity. In contrast, mutating residues from gate region 2 (Lys-159, Leu-163) resulted in a substantial increase in ATPase activity which, however, as demonstrated for variants ArtM(K159I) and ArtM(K159E), is not coupled to transport. Replacing homologous residues in the closely related histidine transporter of *Salmonella enterica* serovar Typhimurium (HisJ-QMP_2) caused different phenotypes. Mutation to isoleucine of HisQ(K163) or HisM(H172), both homologous to ArtM(K159), abolished ATPase activity. The mutations most likely caused a structural change as revealed by limited proteolysis. In contrast, substantial, albeit reduced, enzymatic activity was observed with variants of HisQ(L167 → G) or HisM(L176 → G), both homologous to ArtM(L163). Our study provides the first experimental evidence in favor of a crucial role of residues from the proposed gate region in type I ABC importer function.

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1. Introduction

ATP-binding cassette (ABC)-systems are found in all three domains of life and form one of the largest protein superfamilies of paralogous sequences [1]. The ABC superfamily includes exporters and importers, the latter until recently being confined to prokaryotes but may also exist in yeast, protozoa and plants [2,3], and non-transport ABC-proteins which do not possess transmembrane domains.

Canonical ABC transporters share a common structural organization comprising two transmembrane domains (TMDs) that form the translocation path and two nucleotide-binding domains (NBDs) that hydrolyze ATP. Substrates transported by ABC-transporters are diverse, such as sugars, amino acids, peptides, vitamins, ions, xenobiotics, and even polypeptides, linking ABC transporters to various cellular functions that range from energy supply to osmoregulation, detoxification, and virulence [3,4]. Substrate specificity is accomplished by the TMDs, which display basically no sequence homologies and feature varying numbers of transmembrane helices (4–10) among different ABC transporters.

Prokaryotic canonical ABC importers which are dependent on high-affinity substrate binding proteins (SBP) [5], mediate the uptake of nutrients, osmoprotectants, various growth factors or trace elements [3]. SBPs bind their substrates with high specificity and high affinities. The secondary structural elements of SBPs exhibit common features that consist of two large lobes interconnected by a hinge region. The lobes reside in an 'open' conformation in the absence of ligand and change conformation to a 'closed' form upon trapping their specific substrate ('venus flytrap' model) [5]. SBPs not only capture and accumulate the substrate in proximity to the transporter but also play an important functional role in the catalytic cycle of the transporter [6].

Canonical ABC importers may be subdivided into types I and II [7]. The first type comprises smaller importers featuring a transmembrane core of 10–14 helices. Type II importers on the other hand display larger

Abbreviations: ABC, ATP-binding cassette; DDM, n-dodecyl-β-D-maltopyranoside; His-tag, hexahistidine tag; IPTG, isopropyl-β-D-thio-galactopyranoside; MOPS, 3-(N-morpholino)-propanesulfonic acid; OG, octyl-ß-D-glucopyranoside; PMSF, phenylmethylsulfonylfluoride

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transmembrane domains with up to 20 helices and specificity for metal chelates, heme, and vitamin B_{12}.

To date, crystal structures of two archaeal and four bacterial ABC importers have been solved [8]. Largely based on these structures, but also evidenced by biochemical and biophysical approaches, ABC transporters are thought to function by an 'alternate access' mode [8]. In particular, X-ray structures of the type I maltose transporter of *Escherichia coli* captured in different states added to the concept [9–11]. Accordingly, the TMDs switch from an inward to outward facing state, depending on the nucleotide status of the NBDs. In the absence of nucleotides (apo-state), SBP-dependent ABC importers reside in an inward-facing conformation. Upon concomitant binding of liganded SBP and ATP which cause the NBDs to fully close [12], the TMDs change to an outward facing conformation and the substrate is released to a putative binding site within the transmembrane core which to date has only been identified in the maltose transporter [9]. Subsequent hydrolysis of ATP eventually leads to delivery of the ligand to the cytoplasm and return of the transporter to the apo-state. Although a key step in this process, with the exception of the maltose transporter [13], little is known on how liganded SBP initiates the transport cycle. In particular, how substrate is released and guided to the binding site within the translocation pathway is poorly understood. The structure of the molybdate transporter of *Archaeoglobus fulgidus*, $Mod(BC)_2$ which was solved in the inward facing conformation in complex with its cognate SBP, ModA, revealed the existence of a 'gate' beneath the interface of the TMDs with the binding protein which marks the entrance to the translocation pore [14]. Structurally conserved periplasmic gate regions were also identified within the structures of the maltose ($MalFGK_2$) [9] and the methionine transporter ($MetN_2I_2$) [15] of *E. coli*. The observation, that the structurally related residues in the maltose transporter are close to the internal substrate binding site was taken as further evidence for the above notion. However, to our knowledge, no functional analysis of residues forming the gate has yet been reported. Thus, this lack of information prompted us to investigate the consequences of mutations affecting residues from the gate in the type I transporter for positively charged amino acids from the thermophilic bacterium *Geobacillus stearothermophilus* ArtJ-$(MP)_2$.

The transporter consists of the binding protein, ArtJ, displaying highest binding affinity for arginine [16], and a homodimer each of the transmembrane subunit, ArtM, and the nucleotide-binding subunit, ArtP [17]. ArtM is predicted to span the membrane five times and thus, is one of the smallest TMDs known. Crystal structures of ArtJ in complex with different ligands [17] and of $ArtP_2$ with bound nucleotides [18] have been solved while structural information on the complete transporter is elusive. However, a model of the ArtM dimer using the structure of MetI [19] as template was created and guided the intended study (Fig. 1A). Sequence alignment of ArtM with close relatives revealed conserved amino acid residues within two peptide regions that constitute the proposed gate (Fig. 1B, C). We found that residues from gate region 1 are dispensable for function while replacing residues from region 2 caused a drastic increase in binding protein-dependent ATPase activity. Uptake assays indicate however, that the increase in specific ATPase activity is not coupled to arginine transport. In contrast, replacing homologous residues from region 2 in the TMDs of the related transporter HisJ-QMP_2 from *Salmonella enterica* serovar Typhimurium [20] either abolished ATPase activity or resulted in only moderate reduction of activity. Together, these results strongly suggest a crucial role of residues from region 2 in type I transporter function.

2. Materials and methods

2.1. Bacterial strains and plasmids

Strains and plasmids used in this study are listed in Table 1.

2.2. Protein preparations

$Art(MP)_2$ (wild type and variants) was purified by making use of a His_6-tag engineered to the carboxyl terminus of ArtP as described in Ref. [17]. Briefly, *E. coli* strain BL21(DE3)T1 placI transformed with pRF2 or derivatives was grown in TB-ampicillin/chloramphenicol to an OD_{650} of 0.5 prior to the addition of 0.5 mM IPTG for induction of gene expression. For purification of $Art(MP)_2$, the membrane fraction obtained after cell disruption and ultracentrifugation (1 h at 200,000 ×*g*) was resuspended in 50 mM MOPS-KOH, pH 7, 10% (v/v) glycerol, 0.1 mM PMSF and solubilized with 1.1% DDM. After ultracentrifugation (30 min at 200,000 ×*g*) the supernatant was adjusted to 10 mM ATP and 300 mM NaCl and purified on a TALON matrix (Clontech) in the presence of 0.05% DDM.

ArtJ was overproduced and purified as described in Ref. [16]. Briefly, cells of *E. coli* strain BL21(DE3)T1 (pLysS, pSN1) were grown in LB (Luria Bertani) medium supplemented with ampicillin and chloramphenicol at 37 °C. At an OD_{650} of 0.5, overexpression was induced by adding 0.5 mM IPTG, and growth was continued for 4 h. Cells were harvested, resuspended in 50 mM MOPS-KOH, pH 7, 100 mM NaCl, 0.1 mM PMSF and disintegrated by passage through a French Press. After ultracentrifugation, the supernatant containing His_6-tagged ArtJ was applied to metal affinity chromatography using a TALON resin.

$HisQMP_2$ (wild type and variants) was solubilized from the membrane fraction of *E. coli* strain BL21(DE3)T1 (pLysS) harboring plasmid pVE26 or derivatives by DDM (1.1%) as in Ref. [20] and purified on a TALON resin in the presence of DDM (0.05%) essentially as described for $Art(MP)_2$.

HisJ was purified from the cytosolic fraction of *E. coli* strain BL21(DE3)T1 (pLysS, pSN2) by metal affinity chromatography on a TALON resin essentially as described for ArtJ [16].

2.3. Preparation of proteoliposomes for ATPase assays

Incorporation of $Art(MP)_2$ into liposomes prepared from *G. stearothermophilus* total lipids was carried out as described in Ref. [16]. Briefly, lipids (20 mg) were dried under a stream of nitrogen, slowly redissolved in 1 ml 50 mM MOPS-KOH, pH 7.5, containing 1% OG, and sonicated for 15 min. Subsequently, $Art(MP)_2$ variants (50 μg) were added to 125 μl of the lipid–detergent mixture, resulting in a final volume of 300 μl. Proteoliposomes were formed by removal of detergent by adsorption to Biobeads (100 mg; BioRad, München) at 4 °C overnight. After replacing the beads with a new batch, incubation continued for 2 h. Then, the mixture was centrifuged for 1 min at 10,000 ×*g* to pellet the beads and subsequently, proteoliposomes were recovered by ultracentifugation for 30 min at 220,000 ×*g*, resuspended in 50 mM MOPS-KOH, pH 7.5, and assayed for ATPase activity. Proteoliposomes containing $HisQMP_2$ were essentially prepared alike but using *E. coli* total lipids (Avanti Polar Lipids, USA).

2.4. Preparation of proteoliposomes for transport assays

Proteoliposomes were formed by fast dilution of $Art(MP)_2$ variants (100 μg) mixed with *G. stearothermophilus* lipids (5 mg) sonicated in 50 mM Tris–HCl (pH 7.5), 1% octyl β-D-glucopyranoside, in 30 ml 50 mM Tris–HCl (pH 7.5), 7.5 mM ATP. After ultracentrifugation at 200,000 ×*g* for 1 h, proteoliposomes were resuspended in 400 μl 50 mM Tris–HCl (pH 7.5) and stored on ice until use.

2.5. Site-directed mutagenesis

Site-directed mutagenesis was carried out by using Stratagene's QuikChange or QuikChange Lightning kits according to the manufacturer's instructions.

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