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Structure and elevator mechanism of the Na^+-citrate transporter CitS

Juke S Lolkema[1] and Dirk Jan Slotboom[2]

The recently determined crystal structure of the bacterial Na^+-citrate symporter CitS provides unexpected structural and mechanistic insights. The protein has a fold that has not been seen in other proteins, but the oligomeric state, domain organization and proposed transport mechanism strongly resemble those of the sodium-dicarboxylate symporter vcINDY, and the putative exporters YdaH and MtrF, thus hinting at convergence in structure and function. CitS and the related proteins are predicted to translocate their substrates by an elevator-like mechanism, in which a compact transport domain slides up and down through the membrane while the dimerization domain is stably anchored. Here we review the large body of available biochemical data on CitS in the light of the new crystal structure. We show that the biochemical data are fully consistent with the proposed elevator mechanism, but also demonstrate that the current structural data cannot explain how strict coupling of citrate and Na^+ transport is achieved. We propose a testable model for the coupling mechanism.

Addresses
[1] Molecular Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, The Netherlands
[2] Membrane Enzymology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, The Netherlands

Corresponding author: Slotboom, Dirk Jan (d.j.slotboom@rug.nl)

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Introduction

CitS is a secondary active transporter that translocates citrate in symport with two Na^+ ions across the cytoplasmic membrane of Gram-negative bacteria. CitS of *Klebsiella pneumoniae* was first described in 1986 as the Na^+-coupled transporter responsible for the uptake of citrate in the anaerobic citrate degradation pathway in this pathogen [1]. The protein belongs to the 2-HydroxyCarboxylate Transporter (2HCT) family, which includes a variety of other bacterial transporters for mono-carboxylates, di-carboxylates and tri-carboxylates containing a 2-hydroxy group. These transporters are involved in divers energy conservation pathways such as malolactic fermentation, citrolactic fermentation, oxidative malate decarboxylation, and citrate fermentation. The transporter family includes not only Na^+ symporters but also H^+ symporters and precursor/product exchangers [2].

The 2-HydroxyCarboxylate Transporter (2HCT) family has been predicted to be structurally related to 31 other proteins families that do not share significant sequence similarity [3]. In the MemGen classification of membrane protein structures (Box 1), CitS is found in structural class ST3. Very recently a crystal structure was presented of CitS from *Salmonella enterica*, which shares 92% sequence identity with CitS from *K. pneumoniae* [4••]. The structure of CitS is the first high-resolution crystal structure of a member of the 2HCT family. In addition to CitS, crystal structures are available of members from different protein families in class ST3: the succinate transporter VcINDY in the DASS family [5] and the YdaH and MtrF transporters in the AbgT family [6,7]. All these transporters are dimers, with each protomer consisting of two domains: a transport domain and dimerization domain. Translocation of the substrate likely takes place by an elevator-type mechanism, which involves movement of the transport domain across the membrane relative to the stable dimerization domain (Figure 1).

The energy coupling mechanism, substrate specificity, and structure–function relationships of CitS from *K. pneumoniae* have been studied extensively in the three decades between the discovery of the protein and the recent elucidation of a crystal structure. Here we will interpret the large body of data on CitS from *K. pneumoniae* and related proteins from the 2HCT family in the light of the crystal structure. We show that the mechanistic interpretation of the biochemical and bioinformatics data is greatly helped by the availability of the crystal structure. Conversely, the biochemical data provide compelling evidence for the existence of additional structural states, distinct from the ones observed in the crystals, which are needed to explain the coupling mechanism.

Overall structure

Membrane topology

The crystal structure of CitS is fully consistent with the membrane topology model for the 2HCT family that was deduced from biochemical studies (Figure 1b) [2]. The CitS protomer contains two homologous repeats of five

Box 1 MemGen classification

The MemGen classification of membrane proteins uses hydropathy profile alignments to predict whether different protein families share the same overall structure [31]. For secondary transporters four structural classes were defined: ST1-4. ST1 corresponds to the major facilitator superfamily fold, ST2 to the LeuT fold, ST3 includes the CitS protein and ST4 corresponds to the glutamate transporter fold. It turns out that the four structural classes correspond to different mechanisms of transport [32•,33•]: ST1 uses the rocker switch mechanism, ST2 the rocking bundle, and ST3 and ST4 use two different types of elevator mechanisms where the substrate binding sites are either confined to a single protein domain (ST4) or located at the interface between stable and moving domains (ST3). The power of the MemGen classification has been illustrated by the prediction that the amino acid/polyamine/organocation or APC superfamily has the LeuT fold and belongs to ST2 [34], which was later confirmed by crystal structures [35].

The MemGen classification currently clusters 32 different families of secondary transporters in ST3, and predicts that all these families have related structures [3]. The structures of members of three ST3 families have now been solved: vcINDY from the DASS family, YdaH and MtrF from the AbgT family, and CitS from the 2HCT family. Indeed, vcINDY, YdaH and MtrF share a similar fold [36]. These proteins are homodimers in which each protomer consists of a dimerization domain and a transport domain. Each transport domain can move relative to the stable dimerization domain, like an elevator, and thereby translocate substrate across the membrane. CitS has a similar global domain organization, and also works by an elevator mechanism, but the tertiary structures of the domains are different than the ones in vcINDY, YdaH and MtrF. There is no simple divergent evolutionary pathway (such as 3D domain swapping [37,38•]) to relate the folds of CitS with those of vcINDY and the AbgT proteins. Therefore, we speculate that CitS and vcINDY/AbgT have evolved by convergent evolution to proteins with identical oligomeric states, identical domain organization and very similar membrane topologies, which use the same mechanism of transport, but differ in the details of the structures. The resolution of the hydropathy profile analysis used by MemGen is not high enough to detect the differences in the details of the structures, but qualitatively it is possible to tentatively classify ST3 families as 'vcINDY-like' or 'CitS-like'. For example, the GLTS family of sodium coupled glutamate transporters is likely to have the CitS-fold, consistent with topology studies [39].

Before the crystal structures of ST3 members were solved [2], alternating access in CitS was interpreted in the light of the 'rocker switch' type of mechanism [33•] by analogy to the mechanism of the lactose transporter LacY [40] and the glycerol-P/Pi exchanger GlpT [41], two transporters among a few for which crystal structures were available. The crystal structure of CitS of *S. enterica* proved otherwise; CitS translocates its substrate by an elevator mechanism, which was unheard of until 2009 when such a mechanism was proposed for the first time for the glutamate transporter Glt_{Ph} in structural class ST4 [42].

transmembrane segments (TMSs) each (TMS2-6 and TMS7-11, respectively). The repeats were correctly predicted to have inverted orientations in the membrane and are preceded by the N-terminal TMS1, which is not universally present in all protein families of structural class ST3. Each repeat contains a helical hairpin (HP) embedded in the transmembrane region. The helical hairpin in the N-terminal repeat enters the membrane from the periplasm, the other one from the cytoplasm. A sequence motif GGXG is located at the tip of each of the helical hairpins, and the two motifs meet each other approximately in the middle of the membrane embedded region. The helical hairpins are now denoted HP1 and HP2, but had previously been referred to as reentrant loops or pore loops.

Domain structure

Each sequence repeat contributes to both the transport and the dimerization domain, with TMS2-4 and TMS7-9 together forming the dimerization domain, and TMS5-6, TMS10-11 and HP1-2 constituting the transport domain (Figure 1b). The intertwined domain organization is consistent with experiments on split versions of CitS and the related protein GltS (Box 1). Split proteins consisting of either TMS1-6 or TMS7-11 were stable and active when expressed together in the same cell, but were rapidly degraded when produced separately, indicative of intimately interacting TMSs from both repeats [8,9]. Although the experiments with the split proteins are consistent with the crystal structure, at the time of the experiments the domain organization was incorrectly hypothesized to follow the sequence repeats (see Box 1).

Oligomeric structure

A dimeric state of CitS had been inferred from blue-native PAGE [10,11], single-molecule fluorescence spectroscopy [12], affinity chromatography [13] and electron microscopy experiments. Single particle electron microscopy [11] and electron crystallography [14•,15] revealed dimers of CitS that were oval-shaped when viewed along an axis perpendicular to the membrane plane, in line with top views of the crystal structure (Figure 1a) [4••]. Cross-linking studies placed TMSI close to the dimer interface [16] in agreement with the crystal structure. A side view of CitS obtained from single particle analysis was kidney-shaped with protein mass protruding from the predicted membrane plane on both ends. Likely, the protrusions correspond to the transport domains that stick out of the membrane, which correspond to the outward-facing state seen in the high-resolution crystal structure (Figure 1d). In contrast to the structure determined by X-ray crystallography, in which the transport domains of the two protomers in the dimeric ensemble face opposite sides of the membrane (Figure 1e), in the EM structure the two protomers are in the same state, which likely corresponds to the outward-facing conformation.

2HCT structure–function relationships

Citrate and sodium ion binding sites

Citrate and sodium ions are bound to both the inward and outward facing protomers of CitS in the crystals, but the protein–ligand interactions are better defined in the outward-facing conformation. In the inward facing state citrate and sodium ions are less tightly bound, more hydrated, and seem on their way to be released from