University of Groningen

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

Lolkema, Juke; Slotboom, Dirk

Published in:
Current Opinion in Structural Biology

DOI:
[10.1016/j.sbi.2016.10.004](https://doi.org/10.1016/j.sbi.2016.10.004)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2017

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):
Lolkema, J., & Slotboom, D. (2017). Structure and elevator mechanism of the Na⁺-citrate transporter CitS. *Current Opinion in Structural Biology*, 45, 1-9. <https://doi.org/10.1016/j.sbi.2016.10.004>

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

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

Juke S Lolkema¹ and Dirk Jan Slotboom²

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)

Current Opinion in Structural Biology 2017, 45:1–9

This review comes from a themed issue on **Membranes**

Edited by **Martin Caffrey** and **David Drew**

For a complete overview see the [Issue](#) and the [Editorial](#)

Available online 21st October 2016

<http://dx.doi.org/10.1016/j.sbi.2016.10.004>

0959-440X/© 2016 Elsevier Ltd. All rights reserved.

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 diverse 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-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 Glp_{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 TMS1 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

Figure 1

Overall structure of CitS [4^{**}]. **(a)** Top view along an axis perpendicular to the membrane plane in secondary structure cartoon representation. The transport domains of the two protomers are shown in light and dark green respectively, the citrate molecules in red, and the dimerization domain in light and dark gray. **(b)** Membrane topology of the CitS protomer. Transmembrane segments (TMSs) are numbered 1–11, and the helical hairpins HP1 and HP2. The two helical segments in each hairpin are labeled a and b. Green elements together constitute the transport domain; gray elements the dimerization domain. Yellow circles indicate the positions of native cysteines C398 and C414. **(c)** Side view along an axis parallel to the membrane plane in surface representation. Only one of the transport domains of the dimer is visible and colored green. This domain is in the inward facing conformation. The dimerization domains in pale cyan, citrate (buried) in red. The approximate position of the membrane is indicated by the gray rectangle. **(d)** Same as (c) but now the outward facing transport domain is visible. **(e)** Slice through of the CitS asymmetric dimer in surface representation, with the transport domains in different positions, as observed in the crystal structure [4^{**}]. The transport domains of the two protomers are shown in green, the citrate molecules as red sticks, and the dimerization domains in dark gray. The view is along an axis parallel to the membrane plane. Panels a,c,d and e were created using Pymol.

the site. Nonetheless, the same residues of the transport domain define the binding sites in the inward and outward facing states. The citrate molecule and sodium ions bind close to each other at the tips of hairpins HP1 and HP2 (Figure 2a). The tip surfaces correspond to the essentially conserved sequences 180-PGMGGGNGA-188 (HP1) and 399-MANRGGSG-406 (HP2). The sequence motifs GGNG and GGSG were previously predicted to be at the vertex of the reentrant loops [2] and

indeed are found in the tip regions of the HPs. The Gly residues allow the two tips to be in very close proximity.

The citrate molecule is located at the interface between the transport and dimerization domain, but is bound almost exclusively by the transport domain. The N-terminal ends of helices HP1b and HP2b from the two hairpins point towards each other and sandwich the central 2-hydroxy carboxylate group of the citrate molecule between

Figure 2

Citrate and sodium ion binding sites in CitS. **(a)** Helical hairpins HP1 and HP2 in secondary structure cartoon representation. Protein is colored according to conservation from purple (highly conserved) to cyan (poorly conserved) as calculated by the Consurf server [43]. Arrows indicate the helical dipoles. Citrate in stick representation, sodium ions as purple spheres. Native cysteines 398 and 414 are shown in stick representation. **(b)** Citrate binding site. **(c)** Binding site for Na1. **(d)** Binding site for Na2. All panels were created using Pymol.

them (Figure 2a and b). The partial positive charges from the two helix-dipoles are likely charge-compensated by the carboxylate [17]. The central carboxylate is within hydrogen-bonding distance of the backbone NH groups from N186 and G187 from HP1. The hydroxyl group of citrate interacts exclusively with HP2 via the side chain and backbone NH group of S405. The two outer carboxylates of citrate are bound, respectively, by the side chains of R402, S405 and N186 from the HPs, and R428 from TMS11.

The C-terminal ends of the helices HP1a and HP2a also point towards each other. In this case the partial negative charges from the two helix-dipoles stabilize one of the sodium ions, which is located symmetrically between the helical ends (Figure 2a and c). This sodium ion is coordinated by backbone carbonyls from residues I181 and G183 (HP1) and M399 and N401 (HP2), as well as by

two water molecules. The coordination of the second sodium ion is entirely different. It is not bound symmetrically between the HPs and does not interact with HP1 (Figure 2d). The side chains from N401 and D407 and backbone carbonyls from C398 and G403 in HP2 coordinate the ion as well as the sidechain from S427 in TMS11 and a water molecule. In contrast to the citrate molecule, which is located on the periphery of the transport domain and exposed to the dimerization domain, the sodium ions are completely buried in the transport domain.

Mutational analysis of the binding sites

Mutation of the G403, G404 and G406 in the GGSG motif in HP2 and of G184, G185 and G187 in the GGNG motif in HP1 to Cys either completely inactivated the transporter, or led to severe inhibition (less than 10% remaining activity) [18]. The side chain of G187 points away from the transport domain towards the dimerization

domain. Therefore mutation of this residue may obstruct the elevator-like movement. Mutation of the other glycines probably prevents the two hairpins from adopting the compact structure needed to form the sodium and citrate binding sites. Mutation of Asn186 and Ser405 in the motifs also reduced the citrate transport activity but not as dramatically as the mutations of the glycines [18,19]. These residues contribute to the affinity through their direct interaction with citrate but they are not essential for transport.

Native Cys398 is positioned in the helical segment of HP2a, just before the tip region (Figures 1c and 2c) and its backbone carbonyl coordinates one of the Na⁺ ions (see above). Mutation of Cys398 to Ser did not change the Na⁺ stoichiometry of the transport reaction, but the affinity for Na⁺ was reduced by an order of magnitude, 28 mM versus 3 mM [20]. Possibly, replacement of the thiol group with a hydroxyl group negatively affects the interaction between the carbonyl group and the Na⁺ ion. Mutation of Cys414 that is positioned in the helical segment HP2b close to the cytoplasm (Figures 1c and 3a), reduced the affinity for Na⁺ by two-fold [20]. Since Cys414 is far away from the binding sites, the change in affinity is likely to be an allosteric kinetic effect, for instance by affecting conformations changes in HP2 that are needed during the transport cycle (see below).

One of the few residues outside the helical hairpins that bind the citrate molecule is Arg428 in TMS11 of the transport domain (Figure 2b). Arg428 forms a salt bridge with one of the two outer deprotonated carboxylic acids. Arg428 is highly conserved in the 2HCT family and mutation of Arg428 to Cys rendered CitS inactive, while the conservative mutation to Lys of the corresponding Arg residues of the homologues CitP of *Leuconostoc mesenteroides* and CimH of *Bacillus subtilis* reduced the affinity for citrate by an order of magnitude [2,21,22]. CitP transports many mono-carboxylates, di-carboxylates and tricarboxylates, in contrast to CitS, which transports citrate only [23,24]. A detailed stereoselectivity study of CitP showed that mutation of Arg425 (homologous to Arg428 in CitS) reduced the affinity for the *S* enantiomers of dicarboxylates, but increased the affinity for mono-carboxylates and resulted in a model for the interaction of citrate with the protein (see [2]).

Conformational transitions upon citrate and sodium ion binding to CitS

The elevator movement

The 'alternating access' model for transporters [25] provides a conceptual framework for the mechanistic coupling between solute and co-ion translocation catalyzed by secondary transporters. The kinetic model postulates states with the binding sites exclusively accessible from one side of the membrane and not from the other (inward-facing and outward-facing states). Binding and release of

solute and co-ions to and from the binding sites allows for controlled transitions between the states. The crystal structure of CitS revealed two different conformational states of the transporter and provides strong evidence for an elevator-type mechanism, which leads to alternating access (Figure 1c,d).

Movement of the transport domain relative to the dimerization domain is consistent with experiments, in which the order of the two repeats was swapped [9]. These experiments were done on GltS, which has the same global structure as CitS, but lacks TMS1, and consequently has the N-termini and C-termini located on the same side of the membrane (Box 1). The crystal structure of CitS shows that domain swapping should be possible provided that the linker between the original N-termini and C-termini is long enough. Indeed, a linker of 2 residues resulted in an unstable protein. Probably the short connection caused too much strain to allow for a stable conformation. A linker of 12 residues resulted in a stable but inactive protein. This linker may be long enough to bridge the distance between the two termini, but too short to allow the movement of the transport domain *versus* the dimerization domain. Increasing the linker to 18 residues resulted in an active 'swapped' GltS protein.

Structural states not captured in the crystals

In both the inward and outward facing CitS molecules captured in the crystal structure citrate and sodium ions are present in the binding sites, although the binding appears less tightly in the inward facing state [4^{••}]. Besides these states additional ones must exist: for example a state devoid of citrate and sodium ions that allows the reorientation of empty protein after delivery of the cargo to the cytoplasm, and partially bound states. Partially bound states may include the carrier bound to one or two sodium ions without citrate, or conversely a citrate-bound state in the absence of one or both sodium ions. Because transport of citrate and sodium ions is strictly coupled, the partially bound states should not allow for translocation.

The inward open state

The extent of the structural differences between different states has been addressed experimentally for CitS of *K. pneumoniae* by accessibility studies of the native cysteine residues at positions 398 and 414 in the helical segments of hairpin HP2 that enters the protein from the cytoplasmic side (Figures 1c and 2c [20,26]). In the unloaded state the cysteines were readily accessible from the cytoplasmic side of the membrane for thiol modifying reagents of different size and charge. N-ethylmaleimide (NEM, small and uncharged), 4-Acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid (Amdis, bulky and negatively charged), and methanethiosulfonate (MTSET, positively charged) were all able to react with the cysteines. In contrast, in the presence of saturating concentrations of citrate and

Na⁺-ions, Cys398 and Cys414 were fully protected from modification. Movement of the transport domain relative to the scaffold domain alone cannot explain the differences in accessibility, because the cysteines are completely buried in the transport domain within the crystal structure of CitS. Instead it follows that the structure of the unloaded transport domain itself must be different and more open around the two thiols to explain the increased accessibility to alkylating agents.

Modification of the cysteines by NEM inactivated the transporter, possibly because alkylation of the cysteines prevented HP2 from adopting the compact conformation required for sodium and citrate binding. Alternatively, the modified cysteines may hinder the movement of the transport domain relative to the dimerization domain.

Binding of Na⁺ ions in the absence of citrate also protected the cysteines in HP2 against modification by NEM. Protection required the binding of 2 Na⁺ ions, linking the protection to a transition between states in the kinetic mechanism of the transporter. The presence of citrate in the absence of Na⁺ likewise reduced the reactivity of Cys398, but in this case the protection of the transporter was only partial at saturating concentrations of citrate, and the concentrations of citrate needed to give half the protection were at least three orders of magnitude higher than the kinetic affinity for citrate in the transport reaction when sodium is present. Nonetheless, these data indicate that both citrate and Na⁺ bind to the transporter in the absence of the other, thereby hinting at a random binding mechanism [20]. Protection by citrate was strongly enhanced in the presence of low concentrations of Na⁺ ions indicating that the citrate-bound state and the fully occupied state are not the same conformations, and suggesting that citrate and sodium ions bind cooperatively. Consistently, citrate-induced differences were observed in the low-resolution electron density maps of CitS obtained by electron crystallography when citrate was added in the absence of Na⁺, and much more pronounced density changes were observed upon the addition of citrate in the presence of Na⁺ [14[•]].

The outward open state

AmdS can access a cysteine residue introduced at the position of Ser405 located in the GGSG motif of HP2 from the periplasmic side of the membrane. The alkylation reaction results in complete inactivation of the transporter [18]. Since the reagent is membrane impermeable, the access pathway is likely to be the same as the access pathway for citrate from the periplasm in the outward facing state. The molecular mass of AmdS is more than twice the mass of citrate (190 and 490 Da, respectively), and the former is a much more rigid molecule, suggesting that the binding site has to open up further to the external medium than observed in the crystal structure, possibly by further movement of the

transport domain relative to the dimerization domain, or by disrupting the compact structure of transport domain itself, for example, by lifting periplasmic hairpin HP1, similar to what was observed for the glutamate transporter Glt_{Ph} [27]. This interpretation is supported by the much shorter half time of reaction of AmdS with Cys residues at positions 184, 186 and 187 in periplasmic hairpin HP1 (<1 min) than at position 405 in cytoplasmic hairpin HP2 (~4 min) [18].

How is strict coupling achieved?

In the fully loaded transporter the elevator-like movement of the transport domain relative to the dimerization domain carries the substrate and sodium ions across the membrane, as evidenced by the crystal structure. But how is the translocation of the partially loaded states prevented? And how does the empty transporter return to the outward facing state after delivery of the cargo in the cytoplasm? On the basis of the very different reactivities of Cys398 and Cys414 in the various kinetic states of the transporter we hypothesize a model depicted in Figure 3.

In the fully loaded state, the folding of the transport domain is compact with the helical hairpins fixed by bound citrate and Na⁺. Two conserved arginines at positions 402 and 428 point towards the dimerization domain, but are protected from unfavorable interactions with the hydrophobic surface of the dimerization domain because they are bound to the citrate molecule. The formation of a compact fold with charge-compensated arginines probably is essential for sliding of the transport domain against the dimerization domain, which represents the transition between the outward-facing and inward-facing states in the crystal structure. In the fully loaded compact state, the two Cys residues in HP2 do not react with alkylating agents.

If compactness of the transport domain is essential for movement, it is likely that a similar conformation exists in the absence of citrate and the two Na⁺-ions, because in the alternate access model, the unloaded state should be able to isomerize between inward-facing and outward-facing conformations as well. The aspartate transporters Glt_{Tk} and Glt_{Ph} (ST4, Box 1) and the leucine transporter LeuT (ST2, Box 1) have been crystallized in unloaded states [28[•],29[•],30]. In these transporters the side chain of a residue in the vicinity of the binding site takes over the empty space. In the case of CitS we speculate that either one of the arginine side chains 402 and 428 or both take up this role. Their positive charges may be buried in the empty sodium binding sites in the unloaded state. In this way they may preserve the relative position of the hairpins and the compactness of the structure needed for translocation, and simultaneously prevent exposure of the charged side chains to the hydrophobic dimerization domain. Cross-linking of the two helical hairpins of CitS supports that at least at some point the two are in close

Figure 3

Model for transport by CitS. The interface between the dimerization domain (black curved line) and the transport domain (green), which holds the binding sites, is shown schematically. Citrate as red oval, sodium ions as purple spheres. Cysteines 398 and 414 as yellow circles (not accessible for modification) or yellow stars (accessible). Arginines 402 and 428 are indicated as plain R when buried, and as star when hypothesized to be exposed to the interface (hence blocking the elevator). The possible state with citrate bound in the absence of sodium ions has been omitted from the scheme because it may not be relevant at physiological citrate concentrations.

vicinity in the unloaded state of the transporter [8]. The compact unloaded state is likely to be insensitive to thiol reagents as is the compact fully loaded state. In general, the translocation competent states are protected states.

A second type of unloaded state is needed to account for the dramatic increase in the reactivity of the cysteines in the absence of citrate and Na^+ -ions. In the model depicted in Figure 3, the unloaded, compact state is in dynamic equilibrium with a state in which the arginines have moved away from the Na^+ binding sites leading to disruption of the compact structure, and allowing hairpin HP2 to move away from the binding site. In this way the two Cys residues become accessible for cysteine modifying reagents to enter. In the unloaded, compact state the Cys residues are not accessible, in the unloaded, open state they are.

The unloaded compact state can isomerize between outward-facing and inward-facing conformations, but the unloaded open state does not allow movement of

the elevator for two reasons. First, the helical hairpins are at the interface between transport and dimerization domain and, consequently, movement of the hairpin out of the binding site inhibits the translocation step sterically. Second, the potential energy to slide the arginine residues over the hydrophobic surface of the dimerization domain is too high. In the inward-facing conformation, only cytoplasmic hairpin HP2 can go to the 'open' state, periplasmic hairpin HP1 is stuck at the interface. The same may hold for the outward-facing conformation, which ensures that in either conformation at least one hairpin is closed thereby preventing leakage through a channel-like structure. In the model, CitS is only sensitive to cysteine modifying reagents in the absence of substrates and in the state with the transport domain facing the cytoplasm.

The unloaded open conformation provides the access pathway for citrate and Na^+ -ions to their respective binding sites. The binding site for citrate is severely disrupted since the two hairpins have parted which is

in agreement with the low affinity of the unloaded state for citrate. Binding of the two Na⁺-ions results in a Na⁺-bound, compact state where the reactivity of the cysteines is reduced. The Na⁺ ions stabilize the conformation with the hairpins in close vicinity and the compact conformation around the Cys residues. Importantly, in this state, the transport domain cannot isomerize between the inward-facing and outward-facing conformations. We speculate that movement of the transport domain is still blocked by the sidechains of arginines 402 and 428 that are exposed at the interface between the dimerization and transport domain, preventing the elevator-like movement that would lead to sodium ion leakage. Citrate binds with high affinity to the Na⁺-bound state leading to the fully loaded state in which the arginine sidechains are buried again, and the transport domain can move.

Concluding remark

The recent high-resolution crystal structure provides a structural framework for the interpretation of the large body of biochemical data on the protein collected over the past 30 years. Different kinetic states can now be attributed to structural states, but there are crucial questions remaining. The central one from a structural point of view is how strict coupling is achieved.

Funding

This work was funded by the Netherlands Organisation for Scientific Research (NWO) (Vici grant 865.11.001 to D.J.S.) and the European Research Council (ERC) (Starting Grant 282083 to D.J.S.).

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest

1. Dimroth P, Thomer A: **Citrate transport in *Klebsiella pneumoniae***. *Biol Chem Hoppe-Seyler* 1986, **367**:813-823.
2. Sobczak I, Lolkema JS: **The 2-hydroxycarboxylate transporter family: physiology, structure, and mechanism**. *Microbiol Mol Biol Rev* 2005, **69**:665-695.
3. Lolkema JS, Slotboom DJ: **Classification of 29 families of secondary transport proteins into a single structural class using hydropathy profile analysis**. *J Mol Biol* 2003, **327**:901-909.
4. Wöhlert D, Grötzinger MJ, Kühlbrandt W, Yildiz Ö: **Mechanism of Na⁺-dependent citrate transport from the structure of an asymmetrical CitS dimer**. *Elife* 2015, **4**:e09375.
- The high resolution crystal structure of CitS provides direct structural evidence for an elevator-type mechanism.
5. Mancusso R, Gregorio GG, Liu Q, Wang D-N: **Structure and mechanism of a bacterial sodium-dependent dicarboxylate transporter**. *Nature* 2012, **491**:622-626.
6. Bolla JR, Su C-C, Delmar JA, Radhakrishnan A, Kumar N, Chou T-H, Long F, Rajashankar KR, Yu EW: **Crystal structure of the *Alcanivorax borkumensis* YdaH transporter reveals an unusual topology**. *Nat Commun* 2015, **6**:6874.
7. Su C-C, Bolla JR, Kumar N, Radhakrishnan A, Long F, Delmar JA, Chou T-H, Rajashankar KR, Shafer WM, Yu EW: **Structure and function of *Neisseria gonorrhoeae* MtrF illuminates a class of antimetabolite efflux pumps**. *Cell Rep* 2015, **11**:61-70.
8. Dobrowolski A, Fusetti F, Lolkema JS: **Cross-linking of trans reentrant loops in the Na⁺-citrate transporter CitS of *Klebsiella pneumoniae***. *Biochemistry* 2010, **49**:4509-4515.
9. Dobrowolski A, Lolkema JS: **Evolution of antiparallel two-domain membrane proteins. Swapping domains in the glutamate transporter GltS**. *Biochemistry* 2010, **49**:5972-5974.
10. Heuberger EHML, Veenhoff LM, Duurkens RH, Friesen RHE, Poolman B: **Oligomeric state of membrane transport proteins analyzed with blue native electrophoresis and analytical ultracentrifugation**. *J Mol Biol* 2002, **317**:591-600.
11. Mościcka KB, Krupnik T, Boekema EJ, Lolkema JS: **Projection structure by single-particle electron microscopy of secondary transport proteins GltT, CitS, and GltS**. *Biochemistry* 2009, **48**:6618-6623.
12. Kästner CN, Prummer M, Sick B, Renn A, Wild UP, Dimroth P: **The citrate carrier CitS probed by single-molecule fluorescence spectroscopy**. *Biophys J* 2003, **84**:1651-1659.
13. Pos KM, Bott M, Dimroth P: **Purification of two active fusion proteins of the Na⁺-dependent citrate carrier of *Klebsiella pneumoniae***. *FEBS Lett* 1994, **347**:37-41.
14. Kebbel F, Kurz M, Arheit M, Grütter MG, Stahlberg H: **Structure and substrate-induced conformational changes of the secondary citrate/sodium symporter CitS revealed by electron crystallography**. *Structure* 2013, **21**:1243-1250.
- A low-resolution structure from electron crystallography suggests a random binding mechanism for citrate and sodium ions.
15. Kebbel F, Kurz M, Grütter MG, Stahlberg H: **Projection structure of the secondary citrate/sodium symporter CitS at 6 Å resolution by electron crystallography**. *J Mol Biol* 2012, **418**:117-126.
16. Krupnik T, Dobrowolski A, Lolkema JS: **Cross-linking of dimeric CitS and GltS transport proteins**. *Mol Membr Biol* 2011, **28**:243-253.
17. Hol WG, van Duijnen PT, Berendsen HJ: **The alpha-helix dipole and the properties of proteins**. *Nature* 1978, **273**:443-446.
18. Dobrowolski A, Lolkema JS: **Functional importance of GGXG sequence motifs in putative reentrant loops of 2HCT and ESS transport proteins**. *Biochemistry* 2009, **48**:7448-7456.
19. Kästner CN, Dimroth P, Pos KM: **The Na⁺-dependent citrate carrier of *Klebsiella pneumoniae*: high-level expression and site-directed mutagenesis of asparagine-185 and glutamate-194**. *Arch Microbiol* 2000, **174**:67-73.
20. Sobczak I, Lolkema JS: **Alternating access and a pore-loop structure in the Na⁺-citrate transporter CitS of *Klebsiella pneumoniae***. *J Biol Chem* 2004, **279**:31113-31120.
21. Bandell M, Lolkema JS: **Arg-425 of the citrate transporter CitP is responsible for high affinity binding of di- and tricarboxylates**. *J Biol Chem* 2000, **275**:39130-39136.
22. Krom BP, Lolkema JS: **Conserved residues R420 and Q428 in a cytoplasmic loop of the citrate/malate transporter CimH of *Bacillus subtilis* are accessible from the external face of the membrane**. *Biochemistry* 2003, **42**:467-474.
23. Bandell M, Ansanay V, Rachidi N, Dequin S, Lolkema JS: **Membrane potential-generating malate (MleP) and citrate (CitP) transporters of lactic acid bacteria are homologous proteins. Substrate specificity of the 2-hydroxycarboxylate transporter family**. *J Biol Chem* 1997, **272**:18140-18146.
24. Pudlik AM, Lolkema JS: **Substrate specificity of the citrate transporter CitP of *Lactococcus lactis***. *J Bacteriol* 2012, **194**:3627-3635.
25. Jardetzky O: **Simple allosteric model for membrane pumps**. *Nature* 1966, **211**:969-970.
26. Sobczak I, Lolkema JS: **Accessibility of cysteine residues in a cytoplasmic loop of CitS of *Klebsiella pneumoniae* is controlled by the catalytic state of the transporter**. *Biochemistry* 2003, **42**:9789-9796.

27. Boudker O, Ryan RM, Yernool D, Shimamoto K, Gouaux E: **Coupling substrate and ion binding to extracellular gate of a sodium-dependent aspartate transporter.** *Nature* 2007, **445**:387-393.
28. Jensen S, Guskov A, Rempel S, Hänelt I, Slotboom DJ: **Crystal structure of a substrate-free aspartate transporter.** *Nat Struct Mol Biol* 2013 <http://dx.doi.org/10.1038/nsmb.2663>.
The structural basis for the return of the empty aspartate transporter Glt_{TK} to the outward facing state after delivery of substrate and co-ions in the cytosol.
29. Malinauskaitė L, Said S, Sahin C, Grouleff J, Shahsavari A, Bjerregaard H, Noer P, Severinsen K, Boesen T, Schiøtt B *et al.*: **A conserved leucine occupies the empty substrate site of LeuT in the Na⁺-free return state.** *Nat Commun* 2016, **7**:11673.
The structural basis for the return of the empty leucine transporter leuT to the outward facing state after delivery of substrate and co-ions in the cytosol.
30. Verdon G, Oh S, Serio RN, Boudker O: **Coupled ion binding and structural transitions along the transport cycle of glutamate transporters.** *Elife* 2014, **3**:e02283.
31. Lolkema JS, Slotboom DJ: **Estimation of structural similarity of membrane proteins by hydropathy profile alignment.** *Mol Membr Biol* 1998, **15**:33-42.
32. Slotboom DJ: **Structural and mechanistic insights into prokaryotic energy-coupling factor transporters.** *Nat Rev Microbiol* 2014, **12**:79-87.
Review with an overview of transporter mechanisms.
33. Drew D, Boudker O: **Shared molecular mechanisms of membrane transporters.** *Annu Rev Biochem* 2016, **85**:543-572.
Review with an overview of transporter mechanisms.
34. Lolkema JS, Slotboom DJ: **The major amino acid transporter superfamily has a similar core structure as Na⁺-galactose and Na⁺-leucine transporters.** *Mol Membr Biol* 2008, **25**:567-570.
35. Shaffer PL, Goehring A, Shankaranarayanan A, Gouaux E: **Structure and mechanism of a Na⁺-independent amino acid transporter.** *Science* 2009, **325**:1010-1014.
36. Vergara-Jaque A, Fenollar-Ferrer C, Mulligan C, Mindell JA, Forrest LR: **Family resemblances: a common fold for some dimeric ion-coupled secondary transporters.** *J Gen Physiol* 2015, **146**:423-434.
37. Jaehme M, Guskov A, Slotboom DJ: **Crystal structure of the vitamin B3 transporter PnuC, a full-length SWEET homolog.** *Nat Struct Mol Biol* 2014 <http://dx.doi.org/10.1038/nsmb.2909>.
38. Jaehme M, Guskov A, Slotboom DJ: **The twisted relation between Pnu and SWEET transporters.** *Trends Biochem Sci* 2015, **40**:183-188.
A hypothesis on the occurrence of domain swapping to explain the evolutionary relationship between Pnu and SWEET transporters.
39. Dobrowolski A, Sobczak-Elbourne I, Lolkema JS: **Membrane topology prediction by hydropathy profile alignment: membrane topology of the Na⁺-glutamate transporter GltS.** *Biochemistry* 2007, **46**:2326-2332.
40. Abramson J, Smirnova I, Kasho V, Verner G, Kaback HR, Iwata S: **Structure and mechanism of the lactose permease of *Escherichia coli*.** *Science* 2003, **301**:610-615.
41. Huang Y, Lemieux MJ, Song J, Auer M, Wang D-N: **Structure and mechanism of the glycerol-3-phosphate transporter from *Escherichia coli*.** *Science* 2003, **301**:616-620.
42. Reyes N, Ginter C, Boudker O: **Transport mechanism of a bacterial homologue of glutamate transporters.** *Nature* 2009, **462**:880-885.
43. Landau M, Mayrose I, Rosenberg Y, Glaser F, Martz E, Pupko T, Ben-Tal N: **ConSurf 2005: the projection of evolutionary conservation scores of residues on protein structures.** *Nucleic Acids Res* 2005, **33**:W299-W302.