On the kinetics and conformational dynamics of elevator transporters

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Chapter 3

Transport rate analysis reveals binding order of citrate and Na\(^+\) in CitS from *Klebsiella pneumoniae*

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Chapter 3. Kinetic mechanism of CitS

Abstract

The Na\(^+\)-coupled citrate transporter CitS from Klebsiella pneumoniae is one of the best studied members of the 2-hydroxycarboxylate transporter family. Proteins in this family are involved in diverse energy conservation pathways and are exclusively found in bacteria. The structural data collected over the past couple of years indicated that the protein uses an elevator-type translocation mechanism, which was later confirmed by HS-AFM (Chapter 4). The vast collection of previously published biochemical data corroborates the structural findings and gives insight on the function of conserved amino acids, stoichiometry and substrate specificity. Despite the amount of information, the kinetic mechanism of citrate transport is yet to be determined. In this chapter we measured citrate transport rates as function of a range of citrate and Na\(^+\) concentrations in order to determine the order of binding of the substrates that occurs before transport. Our findings suggest that one Na\(^+\) ion needs to bind to the empty carrier before citrate and a second Na\(^+\) ion can bind in a random step. The forward and reverse transport appear to share the same mechanism.

Introduction

CitS is secondary active transporter and member of the 2-hydroxycarboxylate transporter (2HCT) family. Constituents of this family are found exclusively in bacteria and catalyze either exchange of substrates that contain the 2-hydroxycarboxylate moiety (such as citrate, malate and lactate in MleP and CitP) or cumulative uptake of substrates (such as citrate in CitS) coupled to either H\(^+\) or Na\(^+\) ions [1,2]. Physiologically, transport mediated by 2HCT proteins is connected to several energy conservation pathways, for example citrate fermentation, malolactic fermentation and oxidative malate decarboxylation [3]. CitS from Klebsiella pneumoniae is one of the best characterized members of the 2HCT family. CitS couples the transport of one citrate to two Na\(^+\) ions [4]. The structure of CitS from Salmonella enterica was the first high resolution crystal structure of a member of the 2HCT family [5]. While sequence-based classification grouped CitS in the same structural class as the succinate transporter VcINDY from the DASS family [6,7] and YdaH and MtrF from the AbgT family [8], high resolution structures revealed that there are some differences in the folds of these families. Nonetheless, they are all homodimeric transporters, in which each protomer has two domains: a dimerization domain and a transport domain. The former domain mediates all contacts between the protomers, and forms a stable membrane anchor, while the latter domain is located peripherally, contains most of the binding residues for substrate and cotransported-ions, and can move through the membrane alike an elevator to translocate the cargo. Each protomer in the dimer thus has a separate transporting capability. HS-AFM studies confirmed that the substrate is translocated over the membrane by means of an elevator-like movement, which is performed by the transport domain relatively to the plane of the dimerization domain (Chapter 4). A similar mechanism is used in the glutamate transporter family as shown in Glt\(_{\text{N}}\), Glt and ASCT2 [9–11], but the latter proteins form homotrimeric assemblies, and are not related to CitS in sequence or fold.

In CitS, the protomer is composed of 11 transmembrane segments (TMSs) and two helical hairpin elements (HP1 & HP2, each made up of two alpha helices) [12]. HP1 and 2 together with TMSs 5, 6, 10 and 11 form the transport domain [8]. The tips of HP1 and 2 are in close proximity and form the binding site for citrate and the two Na\(^+\) ions. In the fully loaded state, the citrate molecule fits at the interface of the transport and dimerization domain. The Na1 binding site is located at the C-terminal ends of helices HP1a and HP2a from the two hairpins, while the Na2 binding site only interacts with residues in HP2 [8].
Despite the increasingly detailed structural knowledge, the mechanistic characterization on CitS is lagging behind. To shed light upon the transport mechanism of CitS we aimed to extract information on the kinetic scheme from transport measurement [13]. For the data analysis we used the rapid equilibrium assumption, which facilitates the interpretation, because a generic rate equation has been derived. The equation relates the uptake rate to the concentrations of both Na⁺ and citrate. Through the analysis of $K_m$ and $V_{max}$ it is then possible to obtain mechanistic information.

Methods

CitS was expressed in E.coli strain MC1061 with the L-arabinose inducible vector pBAD24. The cells were grown in LB media supplemented with 100mg/L ampicillin. The expression was induced by addition of 0.05% L-arabinose when the culture reached 0.8 OD600. Three hours after induction the cells were harvested by centrifugation (7000 rpm, 15', 4°C Beckman JLA 9.1000) and resuspended in 20 mM Tris-HCl pH 8. The cells were lysed by means of a cell disruptor cooled to 4°C and operated at 25 PSI. The lysate went through an intermediate centrifugation (15000 RPM, 20', 4°C JA25.50) step to remove cell debris, the supernatant was finally ultracentrifuged (40000 RPM 150', 4°C, Beckman 50.1 Ti) and the pellet was resuspended in 20 mM Tris-HCl pH 8 before storing the membrane vesicles at -80°C.

The membrane vesicles were then added to solubilisation buffer (50 mM Tris-HCl pH 8, 300 mM KCl, 1% DDM), incubated for 45' on a rocking platform at 4°C and finally centrifuged (150000 RPM, 150', 4°C, Beckman 50.1 Ti) and the pellet was resuspended in 20 mM Tris-HCl pH 8 before storing the membrane vesicles at -80°C.

The lipids used to reconstitute CitS contained a 3:1 mixture of E.coli lipid polar extract and egg phosphatidylcholine (PC) (Avanti). Liposomes were homogenized by extruding 11 times through a 400 nm pore size filter and subsequently diluted to 5 mg/mL in 50 mM potassium phosphate buffer pH 7. To allow the insertion of the protein into the bilayer, the lipids were destabilized by step-wise addition of 10% Triton X-100 while scattering was followed at a wavelength of 540 nm. The titration was stopped once the absorption signal decreased to about 60% the maximum value reached. Purified protein was added at a protein:lipid ratio (w/w) of 1:800. The protein lipid mixture was incubated for 30' at RT, and then the detergent was removed by addition of BioBeads in three steps: First 15 mg/mL BioBeads was added followed by incubation for 60' at 4°C, then 19 mg/mL BioBeads was added followed by O/N incubation at 4°C. Finally, 29 mg/mL BioBeads was added followed by 120' incubation at 4°C. BioBeads were then removed and the proteoliposomes were pelleted (80,000 RPM, 25', 4°C, MLA80) and resuspended in 50mM potassium phosphate buffer (pH 7) to a final lipid concentration of 20 mg/ml. The proteoliposomes were subjected to three cycles of freeze-thawing using liquid nitrogen and stored until use.

Affinity measurement with ITC

ITC experiments were performed at 25°C with ITC200 calorimeter (MicroCal). 10 μM of CitS in solution containing 0.15% DM, 50 mM potassium phosphate buffer (pH 7), 100 mM KCl, 200 mM NaCl and were loaded into the ITC cell and titrated with 1 μL injections with a solution of 500 μM citrate, 50 mM potassium phosphate buffer pH 7, 100 mM KCl, 200 mM NaCl and 0.15% DM. Data were analyzed with ORIGIN-based software provided by MicroCal.

Uptake rates were measured at varying [Na⁺] and [Citrate]

The luminal buffer in each proteoliposome preparation was changed to 10mM potassium phosphate buffer (pH 7) and 200mM KCl. The proteoliposomes were first pelleted (80,000 RPM, 25', 4°C, MLA80) and then resuspended in the desired luminal buffer. Three freeze-thaw cycles were performed in order to allow the exchange of buffer in the luminal area of the proteoliposomes. The solution was then extruded 11 times through a filter with 400-nm pore size in order to obtain homogeneously sized uni-lamellar vesicles which were pelleted (80000 RPM, 25', 4°C, TLA100.3) and resuspended to a final lipid concentration of 100 mg/mL. Steady state rates were measured at different concentrations of substrate and co-ions. An array of conditions was prepared as shown in table 1. The blank measurement for each condition was measured by pipetting
For secondary active transporters, classical enzymology methods can be used to determine the kinetic mechanism, and therefore the binding order for the substrates. The initial uptake rates of citrate transport catalyzed by purified and reconstituted CitS into proteoliposomes were determined as a function of citrate and Na⁺ concentrations in the external buffer. The luminal concentrations of substrate Na⁺ at the onset of the experiment were negligible, and the initial concentrations used were sufficient to extract sufficient information on the kinetic mechanism. Na⁺ citrate made the noise too high. The range of concentrations used was ample high enough to extract sufficient information on the kinetic mechanism. Na⁺.

The initial transport rates were determined at Na⁺ concentrations ranging from 50 μM to 200 mM (Table 1). The chosen concentrations allowed for a sufficiently wide window to obtain accurate transport rate measurements. Rates at concentration higher than 100 μM of citrate could not be accurately determined for any Na⁺.

Table 1: Initial rates of aspartate uptake at indicated concentration combinations of citrate and Na⁺. In parenthesis the concentration of choline chloride used in the external reaction buffer to balance the ionic and osmotic strength. The error indicates the standard error of the mean in the triplicate experiment.

<table>
<thead>
<tr>
<th>[Cit] → [Na⁺]</th>
<th>0.5 μM</th>
<th>2.5 μM</th>
<th>5 μM</th>
<th>10 μM</th>
<th>25 μM</th>
<th>50 μM</th>
<th>100 μM</th>
</tr>
</thead>
<tbody>
<tr>
<td>(0.5 mM)</td>
<td>5.8 ± 1.0</td>
<td>13.3 ± 0.1</td>
<td>3.4 ± 0.6</td>
<td>2.5 ± 0.7</td>
<td>2.7 ± 0.9</td>
<td>1.2 ± 0.4</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td>(1 mM)</td>
<td>0.3 ± 0.1</td>
<td>0.7 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>3.5 ± 0.3</td>
<td>3.8 ± 0.2</td>
<td>6.0 ± 0.3</td>
<td>13.9 ± 0.2</td>
</tr>
<tr>
<td>(1.5 mM)</td>
<td>0.8 ± 0.1</td>
<td>1.6 ± 0.1</td>
<td>3.7 ± 0.1</td>
<td>6.3 ± 0.1</td>
<td>12.8 ± 0.1</td>
<td>25.8 ± 0.4</td>
<td>38.4 ± 0.4</td>
</tr>
<tr>
<td>(2 mM)</td>
<td>0.8 ± 0.1</td>
<td>1.6 ± 0.1</td>
<td>3.7 ± 0.1</td>
<td>6.3 ± 0.1</td>
<td>12.8 ± 0.1</td>
<td>25.8 ± 0.4</td>
<td>38.4 ± 0.4</td>
</tr>
<tr>
<td>(5 mM)</td>
<td>1.7 ± 0.1</td>
<td>3.4 ± 0.1</td>
<td>7.9 ± 0.1</td>
<td>10.8 ± 0.1</td>
<td>20.9 ± 0.1</td>
<td>28.4 ± 0.1</td>
<td>36.4 ± 0.1</td>
</tr>
<tr>
<td>(7.5 mM)</td>
<td>6.8 ± 0.1</td>
<td>17.8 ± 0.1</td>
<td>27.8 ± 0.1</td>
<td>34.2 ± 0.1</td>
<td>54.6 ± 0.1</td>
<td>58.8 ± 0.1</td>
<td>82.4 ± 0.1</td>
</tr>
<tr>
<td>(10 mM)</td>
<td>8.5 ± 0.1</td>
<td>21.7 ± 0.1</td>
<td>37.7 ± 0.1</td>
<td>42.5 ± 0.1</td>
<td>58.3 ± 0.1</td>
<td>76.0 ± 0.1</td>
<td>89.8 ± 0.1</td>
</tr>
<tr>
<td>(25 mM)</td>
<td>9.0 ± 0.1</td>
<td>23.3 ± 0.1</td>
<td>42.8 ± 0.1</td>
<td>63.6 ± 0.1</td>
<td>69.8 ± 0.1</td>
<td>104.6 ± 0.1</td>
<td>115.9 ± 0.1</td>
</tr>
<tr>
<td>(50 mM)</td>
<td>8.9 ± 0.1</td>
<td>23.3 ± 0.1</td>
<td>42.8 ± 0.1</td>
<td>63.6 ± 0.1</td>
<td>69.8 ± 0.1</td>
<td>104.6 ± 0.1</td>
<td>115.9 ± 0.1</td>
</tr>
<tr>
<td>(100 mM)</td>
<td>8.2 ± 0.1</td>
<td>23.3 ± 0.1</td>
<td>42.8 ± 0.1</td>
<td>63.6 ± 0.1</td>
<td>69.8 ± 0.1</td>
<td>104.6 ± 0.1</td>
<td>115.9 ± 0.1</td>
</tr>
<tr>
<td>(200 mM)</td>
<td>11.3 ± 0.1</td>
<td>23.9 ± 0.1</td>
<td>42.8 ± 0.1</td>
<td>63.6 ± 0.1</td>
<td>69.8 ± 0.1</td>
<td>104.6 ± 0.1</td>
<td>115.9 ± 0.1</td>
</tr>
<tr>
<td>(300 mM)</td>
<td>11.3 ± 0.1</td>
<td>23.9 ± 0.1</td>
<td>42.8 ± 0.1</td>
<td>63.6 ± 0.1</td>
<td>69.8 ± 0.1</td>
<td>104.6 ± 0.1</td>
<td>115.9 ± 0.1</td>
</tr>
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</table>
concentrations higher than 200 mM were not used because the rate of citrate transport were constant already at Na⁺ concentration of ~100 mM for all citrate concentrations.

In Table 1 are displayed the initial rates obtained for each condition. The columns of the matrix contain the initial citrate transport rates at fixed citrate concentration, but varying Na⁺ concentration. The rates plotted as a function of Na⁺ concentration show a sigmoidal dependence (Figure 1A). Fitting the Hill equation to the data allowed for determination of $v_{\text{max}}^{\text{Na(app)}}$ and $K_{M}^{\text{Na(app)}}$ and $n_{\text{Hill(app)}}$. The superscript “Na” indicates sodium dependent measurements and “apparent” indicates that the measurements were done at a fixed citrate concentrations.

The rows of this matrix contain the rates of citrate transport at a fixed Na⁺ concentration, but varying citrate concentrations. When those rates are plotted as a function of the citrate concentration, an hyperbolic dependency can be observed (Figure 1B) which is consistent with the general rate equation (Chapter 1) [13] and allows to extrapolate $v_{\text{max}}^{\text{Cit(app)}}$ and $K_{M}^{\text{Cit(app)}}$. The superscript “Cit” indicates citrate-dependent measurements at one fixed sodium concentration (hence “apparent”).

The kinetic mechanism can be deduced by analyzing the apparent maximal rates ($v_{\text{max}}^{\text{Cit(app)}}$ and $v_{\text{max}}^{\text{Na(app)}}$) and apparent affinities for citrate ($K_{M}^{\text{Cit(app)}}$) which can normally be determined with high accuracy. Theoretically the Na⁺-citrate coupling stoichiometry can also be extracted from the same dataset, but this is often unfeasible due to the error in the uptake experiments using radiolabeled citrate. Therefore, in practice, knowing the transport stoichiometry is required. For this analysis the stoichiometry of 2 Na⁺:1 citrate was used, as determined by Lolkema et al. [4].

Differently from Glt₉ (Chapter 2), we chose to use the rapid equilibrium approach to analyze the data [13] because the $K_{M}$ values obtained from the transport assay are comparable with the $K_{M}$ measurements obtained by ITC (Figure S2) which are 5.64 ± 1.35 μM and 1.24 ± 1.25 μM respectively at saturating Na⁺ concentrations.
\( v_{\text{max}} \) analysis

The apparent maximal rate in citrate dependent measurements \((v_{\text{max}} \text{Cit}(\text{app}))\) can be expressed as a function of the \( \text{Na}^+ \) concentration [4]:

\[
v_{\text{max}} \text{Cit}(\text{app}) = v_{\text{max}} \frac{[\text{Na}^+]^2}{r + x[\text{Na}^+] + [\text{Na}^+]^2} \tag{1}
\]

in which \( r \) and \( s \) are parameters that depend on the kinetic model, and \( v_{\text{max}} \) is the maximal attainable rate at high sodium and citrate concentration.

Conversely, the apparent maximal rate in \( \text{Na}^+ \)-dependent measurements \((v_{\text{max}} \text{Na}(\text{app}))\) can be expressed as a function of the citrate concentration [4]:

\[
v_{\text{max}} \text{Na}(\text{app}) = v_{\text{max}} \frac{[\text{Ci}]}{pK_m^{\text{Na}} + [\text{Ci}]} \tag{2}
\]

in which \( p \) is a model-dependent parameter, and again \( v_{\text{max}} \) is the maximal attainable rate at high sodium and aspartate concentration.

The apparent maximal rates \((v_{\text{max}} \text{Cit}(\text{app}))\) and \((v_{\text{max}} \text{Na}(\text{app}))\) can be plotted as a function of the fixed substrate concentration [13]. The analysis of these functions offers a qualitative outcome, for which there are three possible cases: (i) \( v_{\text{max}} \text{Cit}(\text{app}) \) is dependent on the concentration of \( \text{Na}^+ \), but \( v_{\text{max}} \text{Na}(\text{app}) \) is not dependent on the concentration of citrate; (ii) \( v_{\text{max}} \text{Cit}(\text{app}) \) is not dependent on the concentration of \( \text{Na}^+ \) but \( v_{\text{max}} \text{Na}(\text{app}) \) on the citrate concentration; (iii) both are dependent on each other.

When \( v_{\text{max}} \text{Cit}(\text{app}) \) is independent of the \( \text{Na}^+ \) concentration, but \( v_{\text{max}} \text{Na}(\text{app}) \) is dependent of the citrate concentration, the kinetic mechanism has an obligate last binding step involving citrate. Conversely, when \( v_{\text{max}} \text{Na}(\text{app}) \) is independent of the citrate concentration, but \( v_{\text{max}} \text{Cit}(\text{app}) \) is dependent of \( \text{Na}^+ \) concentration, the last binding step of the kinetic mechanism involves the obligate binding of \( \text{Na}^+ \). When both \( v_{\text{max}} \) are dependent of either citrate or \( \text{Na}^+ \), the last step of the kinetic mechanism concerns the random binding of \( \text{Na}^+ \) or citrate. Our data (Figure 2A) show that \( v_{\text{max}} \text{Cit}(\text{app}) \) varies with the \( \text{Na}^+ \) concentration, which excludes the obligatory binding of citrate in the last step. It is important to stress that this qualitative analysis can be used only if the rapid equilibrium assumption is valid. Therefore, it was not possible to use it in Chapter 2 for the analysis of aspartate transport by GltTk. The shape of the curve resulting from equation 2 also carries information about the number of \( \text{Na}^+ \) that bind in the final step. If a single \( \text{Na}^+ \) binds last the function will describe a hyperbola, instead when more than one \( \text{Na}^+ \) is bound in the last step the function will describe a sigmoidal curve. However, this kind of analysis is difficult to determine univocally due to the inherent experimental error in the transport experiments. Since a hyperbolic function fits well to the data we tentatively conclude that one \( \text{Na}^+ \) binds in the last step. This conclusion also corroborates the finding from the \( K_m \) analysis (see below). The analysis of the apparent maximal rates for \( \text{Na}^+ \) dependent uptakes shows a dependency on the citrate concentration (Figure 2B), excluding the obligatory binding of \( \text{Na}^+ \) as last step. Combining the results for the analysis of \( v_{\text{max}} \text{Cit}(\text{app}) \) and \( v_{\text{max}} \text{Na}(\text{app}) \), we conclude that the final step in the formation of CitS fully loaded state is a random binding of citrate or \( \text{Na}^+ \).

![Figure 2: (A) Dependence of the maximal citrate transport rate \( v_{\text{max}} \text{Cit}(\text{app}) \) on the concentration of \( \text{Na}^+ \) and (B) dependence of the maximal citrate transport rate \( v_{\text{max}} \text{Na}(\text{app}) \) on the concentration of citrate presented in figure 2. Both lines represents the fit of the Michaelis-Menten equation.](image-url)
**K_M analysis**

Apparent affinity measurements for substrate-dependent uptakes (K_M(app) values) also contain information that helps to distinguish between different kinetic mechanisms. The apparent affinity for citrate is dependent on the concentration of Na⁺ for all kinetic schemes except for fully random mechanisms, where K_M(app) is constant. When the apparent affinity constants are plotted against the concentration of Na⁺ in a double logarithmic plot the values asymptotically approach linear relations in both the high and now Na⁺ concentration extremes [13]. The negative of the slope in the low concentration domain indicates the number of Na⁺ ions that bind before the citrate, whereas the negative of the slope at high Na⁺ concentration indicates the number of Na⁺ ions that bind after the citrate. It is important to note that Na⁺ ions that bind in a random order in the same step as the citrate are not accounted in this number, and that this analysis is valid only within the framework of the rapid equilibrium assumption.

Figure 3 shows the plot of log(K_M(app)) vs log[Na⁺]. The slope of linear regression of the data in the low Na⁺ concentration area is approaching -1. A more precise reading of this slope would require additional data points in the low Na⁺ concentration range, however was not possible because the uptake rates were too low to be determined accurately. At high Na⁺ concentrations, the slope levels off close to zero, indicating that there is no obligatory binding event after citrate. These results are in agreement with the conclusion from the analysis of apparent maximal rates that the last step is the random binding of citrate and one Na⁺, moreover the results from the apparent K_M analysis indicates that the last step is preceded by an ordered step involving only one Na⁺.

**Right side out transport**

The observations made to this point show that the last step of the kinetic mechanism is a random binding of citrate or Na⁺. However, there is a potential complication, because the proteoliposome system most likely will contain CitS transporters in two membrane orientations (right-side out and inside out). This is the result of the reconstitution process, which often leads to a random membrane orientation, which could affect the interpretation of our data. For instance the same experimental outcome (with apparent random binding of Na⁺ and citrate in the last step) can also occur if transport of citrate takes place via two different kinetic mechanisms for forward and reverse transport. So far there is no experimental evidence that the forward and reverse transport reactions use different kinetic mechanisms, however it is

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**Figure 3**: Dependence of the apparent affinities for citrate on the Na⁺ concentration in a double logarithmic scale. The lines represent linear fits at low and high [Na⁺] regimes. The data points represent the average of triplicate measurements and the error bars represent the standard error of the mean.

**Figure 4**: (A) Dependence of the maximal citrate transport rate v_max(Cit) (app) on the concentration of Na⁺ and (B) dependence of the maximal citrate transport rate v_max(Na) (app) on the concentration of citrate by AMdS inhibited CitS. Both lines represent the fit of the Michaelis-Menten equation.
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necessary to account for this possibility. To determine if the kinetic mechanism of CitS depends on the orientation of the protein in liposomes we inactivated the inside-out oriented transporters by cysteine modification using the membrane-impermeable maleimide molecule AMdiS as described by Sobczak and colleagues [15]. Since the information about random or ordered binding in the last step is extracted from \( v_{\text{max}}^{\text{Na}}(\text{app}) \) and \( v_{\text{max}}^{\text{Cit}}(\text{app}) \) measurements (see above), we conducted experiments to test whether \( v_{\text{max}}(\text{app}) \) still depended on the citrate concentration, and whether \( v_{\text{max}}^{\text{Cit}}(\text{app}) \) still depended on the Na\(^+\) concentration when the inside-out oriented molecules were inactivated by the cysteine modification via AMdiS. Indeed the \( v_{\text{max}}(\text{app}) \) values still varied with the concentrations of the co-substrate, confirming random binding of Na\(^+\) or citrate in the last step for the right-side-out oriented transporters (Fig. 4AB).

Conclusions

The analysis presented here is, to the best of our knowledge, the most comprehensive kinetic study on CitS from Klebsiella pneumoniae. The rapid equilibrium approach was applied to analyse the initial rates of citrate transport at different concentrations of citrate and Na\(^+\) to determine the kinetic scheme [13]. The use of the rapid equilibrium approach (instead of the more complicated steady-state analysis, see chapter 2) was justified because the \( K_v \) values and \( K_i \) values for citrate at 200 mM NaCl are comparable, which implies that the contribution of \( k_{\text{cat}} \) is negligible when compared to the off-rate \( (k_\text{off}) \). It must be stressed however, that it proved difficult to measure \( K_v \) values accurately by ITC. Nonetheless, it was clear that the \( K_v \) and \( K_i \) difference were both in the low micromolar range, in contrast the \( K_v \) and \( K_i \) values measured for aspartate binding and transport by GltTk which diverged by an order of magnitude (Chapter 2). Hence the approximations for the rapid equilibrium were adopted. It must also be noted that we intended to measure the \( K_v \) for citrate binding over a larger range of sodium concentrations, to establish more firmly that the rapid equilibrium assumption was valid. However the sensitivity of the ITC method was not good enough to determine \( K_v \) values at concentrations of Na\(^+\) below 200 mM.

The dependencies of \( v_{\text{max}}^{\text{Cit}}(\text{app}) \) and \( v_{\text{max}}^{\text{Na}}(\text{app}) \) on the concentrations of Na\(^+\) and citrate respectively (Figure 2AB) indicate that there is a random binding event between citrate and at least one Na\(^+\) as last step in order to reach the productive state of the transporter, with all the cargo loaded. The plot of \( \log(K_m^{\text{Cit}}(\text{app})) \) vs \( \log([\text{Na}^+]) \) (Figure 3) can be used to extrapolate how many Na\(^+\) ions reach the binding site before and after the citrate binding event. The double logarithmic plot shows that \( K_m^{\text{Cit}}(\text{app}) \) value diminishes linearly with a slope approaching -1 in the extreme of low [Na\(^+\)] indicating that one Na\(^+\) ion binds before the citrate-Na\(^+\) random binding step, while in the extreme of the high concentration regime the curve levels off to a slope of zero. Since co-ions that bind randomly with the substrate in the last step do not affect the slope of the double log plot, the \( K_m \) measurements are consistent with the random nature of the last binding step before the formation of the productive state. With this method it is not possible to discriminate which of the two sites found in the crystal structures is occupied by the first Na\(^+\), but a mechanism can be inferred. The binding site for Na\(^+\)_2 is likely to be the first ion to bind as it is buried deeper within the two hairpins. Subsequently the random binding occurs between citrate and the sodium ion that occupies Na\(^+\)_1. The latter two binding sites are not hindered by each other and occupy opposite ends of the hairpin cleft (Figure 5).

In this respect CitS differs from GltTk, because binding of all sodium ions to the latter protein would prevent access for the substrate aspartate.

Figure 5: View of the citrate and Na\(^+\) binding site of the outward-facing transport domain of SeCitS. The Na\(^+\)_1 ion in Na\(^+\)_1 (purple) is coordinated by both HP loops and is buried within the domain. The citrate molecule (pink) and the Na\(^+\) in Na\(^+\)_2 (purple) is only coordinated by residues in HP2.
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Supplementary data & Tables

Figure S1: Time course of citrate uptake into proteoliposomes with reconstituted CitS to determine the time for which the initial rate is constant. The uptake was measured at vmax conditions (200 mM NaCl and 200 μM citrate). The experiment was done in duplicate with the error representing the standard deviation. We concluded that the transport rate is constant over the first 20 seconds of the experiment.

References


Supplementary data & Tables