nature research

Peer Review File

Manuscript Title: Structure and inhibition mechanism of the human citrate transporter NaCT

Reviewer Comments & Author Rebuttals

Reviewer Reports on the Initial Version:

Referees' comments:

Referee #1 (Remarks to the Author):

The manuscript by Sauer et al titled "Structure and inhibition mechanism of the human citrate transporter NaCT" reports structures of human NaCT in the presence of a substrate citrate or an inhibitor PF2. The structures led to identification of two Na ion binding sites, the citrate binding site, and the PF2 binding site. The authors showed technical dexterity in expressing and stabilizing the human NaCT protein and in solving its structures by cryoEM. Notably, the molecular weight of the dimer is modest in cryoEM standard and the authors collected data in tilt series to overcome preferred orientation of NaCT particles on croyEM grids.

The human NaCT structures provide a clear explanation for the specificity of certain inhibitors to a subfamily of NaCT and will almost certainly help the effort of targeting NaCT with small molecules. However, the structures and the follow up mutational studies do not give substantial new information in terms of Na ion binding, citrate binding, or the mechanism of coupled transport. These structures also failed to explain the stoichiometry of Na and citrate unique to human NaCT. In addition, the anisotropy in data very likely compromised the quality of densities for Na and substrate.

Below are a few minor comments that require only revision of figures and text.

Density for each helix should be shown individually with the structural model. At the very least, densities and structural models should be shown for key structural elements such as helices 4a, 4b, 5a, 5b, 9a, 9b, 10a and 10b, and loops such as HPin, HPout, L5ab and L10ab.

Citrate densities should be shown in a similar way as these of Na ions or PF2: densities of residues that form the binding site and densities of citrate should be shown simultaneously at the same sigma level.

The densities of Na ions shown in Figure 2a and b are weak, given that Na is much heavier than C. Can the authors comment on whether the electrostatics of the Na ion binding sites are sufficient to accommodate a cation? In many transporters and ion channels, Na ion binding sites have explicit charges such as glutamate or aspartate.

Could the authors indicate whether Na ions were included in the calculation when they show the electrostatic surface for the inhibitor binding site i Figure 3c? Given that PF2 has two negative charges while citrate has three, is there any difference in their requirement for Na? I am not asking for more experiments, but do authors know if citrate binding is dependent on Na ions, and whether PF2 binding is less dependent on Na?

Can the authors show the electrostatic surface of the citrate binding pocket with and without the presence of the two Na ions? This would help readers understand whether Na ions change the electrostatic surface of the binding pocket. Can the authors comment on whether Na ions can bind to and neutralize the carboxylate on citrate?

The structural model for the tert-butyl group on PF2 seems odd (Figure 3c and Extended Figure 7f. The central carbon does not seem to be a tetrahedron.

Referee #2 (Remarks to the Author):

In their manuscript the authors describe the architecture of the human citrate transporter SLC13A5 (NaCT), the structural basis for substrate recognition and its inhibition by the selective inhibitor PF2. The study is well performed and provides insight into the functional mechanism and pharmacology of an important human membrane transport system. Due to the preferred orientation of particles, the structure determination process was challenging. However, the authors overcame this obstacle by data collection at different tilt angles, which resulted in maps of high quality. In light of the bio-medical importance of the investigated protein and the general conclusiveness of results I regard the study as candidate for publication in Nature.
I have several comments the authors should address:
• In light of the data anisotropy and the unusual decay of the Fourier shell correlation shown in Extended Data Fig. 4 f,g, the drop of the correlation coefficient below the 0.143 threshold is a poor indicator for the effective resolution of the cryo-EM density. The authors have acknowledged this by truncating the resolution to 3.00 Å. As even this resolution might be overestimated (as evidenced in the density shown in Fig. 1b which either shows features of anisotropy or over-refinement at its periphery), I suggest that the authors better document the quality of the map in different regions of the protein. This could be done by plotting the local resolution on the map shown in Fig. 1b and by showing density in different regions of the protein including the substrate binding site.

• In Extended Data Fig.4 f and 6d, the FSC curve for the corrected masked map shows a dip around 4.5A resolution. This can happen during data processing in cryoSPARC as dynamic masking is applied during refinement. As a control, I suggest to either provide a static mask manually (e.g. after being created in Chimera) or to alternatively lower the threshold for dynamic masking. It would be also good to have an inset with a final mask provided to make sure that no part of the protein density is located outside of the mask.

• The evidence for observing bound Na+ ions in Fig. 2a and b is weak.

• I would be helpful to provide a better representation of the substrate binding site and the surrounding density shown in Fig. 2c. Although the argument that the density of citrate might be compromised by the poor scattering properties of carboxylate groups is credible, it would still be interesting to provide a better view of the region. Similarly, the panels showing the density and interaction mode of the inhibitor could be improved.

• Does the inhibitor lock the transporter in its inward-facing conformation? Due to the negative charge I expect the inhibitor not to be membrane permeable. Is it known how it would access its binding site?

Minor:

• Why was the purification carried out in the presence of Li+? Is Li+ expected to occupy Na+ binding sites even if its concentration is 10-times lower than that of Na+?

• What is the net-charge of citrate under physiological conditions?

• I believe citrate transport is electrogenic, is the charge per transport cycle known?

• It is not clear to me what the mesh in Fig. 3b exactly shows, how the coulombic potential was calculated and at which level the mesh is contoured.

• How often have experiments in Fig. 1 a been carried out and what was the error between different experiments.

Referee #3 (Remarks to the Author):

In this study Sauer et al. determined cryo-electron microscopy structures of human NaCT in complex with citrate and with an inhibitor in order to better understand the structural basis of substrate binding and inhibition mechanism. These structures reveal how NaCT recognizes citrate, how the inhibitor arrests the protein's transport cycle and why it selectively inhibits NaCT over two homologous dicarboxylate transporters. From these results the authors conclude that the NaCT structures provide a framework for understanding how various mutations abolish NaCT's transport activity and thereby cause SLC13A5 epilepsy in newborns.

Comments

This is a carefully performed, well-written study by an established group of structural biologists, which has determined the cryo-EM structures of human NACT in complex with citrate and an inhibitor. Given the role of the NaCT transporter in SLC13A5 epilepsy and as a potential anti-obesity drug this manuscript should be of general interest.

My only suggestion would be to discuss how alterations in citrate transport in the CNS might lead to epilepsy and how reductions in the NaCT transporter might result in alterations in energy metabolism and serve as a target for anti-obesity drugs. This would increase the appeal of this article to non-structural biologists.

Author Rebuttals to Initial Comments:

We are grateful to the three reviewers for their time and effort in reading the manuscript and providing insightful comments. We have made changes based upon their suggestions, resulting in a clearer and sharper manuscript.

We appreciate that the reviewers agree that the human NaCT structures, the first of any mammalian SLC13 transporters, provide a framework for understanding the SLC13A5-Epilepsy mutations and, at the same time, establish a structural basis for the design and optimization of small molecule therapeutics. We also appreciate that they recognize both the technical challenges with the preferred orientation problem and the advantage of the specimen tilt approach we used in solving the structure of such a small membrane protein.

Following the reviewers' comments and suggestions, we have revised the text and remade many of the figures. Our point-to-point response to their comments is listed below. To make the response clearer, we have taken the liberty of labeling their comments by numbers as, 1-1, 1-2, … 2-1, 2-1, … etc.

Referee #1 (Remarks to the Author):

The manuscript by Sauer et al titled ???Structure and inhibition mechanism of the human citrate transporter NaCT??? reports structures of human NaCT in the presence of a substrate citrate or an inhibitor PF2. The structures led to identification of two Na ion binding sites, the citrate binding site, and the PF2 binding site. The authors showed technical dexterity in expressing and stabilizing the human NaCT protein and in solving its structures by cryoEM. Notably, the molecular weight of the dimer is modest in cryoEM standard and the authors collected data in tilt series to overcome preferred orientation of NaCT particles on croyEM grids.

1-1. The human NaCT structures provide a clear explanation for the specificity of certain inhibitors to a subfamily of NaCT and will almost certainly help the effort of targeting NaCT with small molecules.

However, the structures and the follow up mutational studies do not give substantial new information in terms of Na ion binding, citrate binding, or the mechanism of coupled transport. These structures also failed to explain the stoichiometry of Na and citrate unique to human NaCT. In addition, the anisotropy in data very likely compromised the quality of densities for Na and substrate.

This manuscript has focused on the overall structure of NaCT and the inhibition mechanism and specificity of the PF2 inhibitor, instead of sodium or citrate binding, or Na+/citrate coupling. As this is the first report of a mammalian NaCT structure, we have focused on its role in human physiology and pharmacology. Our structure of human NaCT provides a needed framework for the understanding of the role of various mutations that cause SLC13A5-Epilepsy in newborns. Furthermore, our high- resolution description of PF2 binding explains its inhibition mechanism, and the binding pocket structure will directly guide the optimization of potency and selectivity of PF2 into an anti-obesity drug.

Regarding sodium and citrate binding, their stoichiometry, and the mechanism of coupled transport, we have not explored these topics in detail within the current report due to both technical challenges and manuscript scope. The competition from Li^+ in the buffer, which was essential to stabilize this human protein for structural determination, likely reduced the densities for Na^+s. Similarly, the -3 charged of citrate made the substrate particularly sensitive to electron radiation damage and yielded a lower citrate density in the map, compared with small molecule substrates of similar sizes with lese negative charges. These factors limit our ability to discuss sodium and citrate binding in detail. Regarding the stoichiometry of transport, while there are always weaker densities at lower map sigma levels, assignment of any of these as sodium without additional evidence would be speculative.

We agree with the reviewer that because of the weak density for citrate its coordination is not well defined. We have therefore removed the contact map for citrate from Figure 2 and shortened the related text. Instead, we have focused on the

location of citrate. Establishing this location is important, as it defines the competitive nature of the PF2 inhibitor

Finally, to understand citrate and Na^+ coupling would require a structure of NaCT in the presence of Na^+ but without citrate or PF2. However, we have found that substrate-free NaCT is unstable *in vitro*, and were unable to purify the protein in the absence of citrate or PF2. Fortunately, readers interested in substrate/Na^+ coupling of this family of proteins can consult our paper on bacterial NaCT homologs, in which we were able to purify and solve structures of bacterial proteins in the absence of a substrate in order to describe substrate coupling (Sauer, et al., eLife, (2020), 9, e61350). In future studies, we will examine the details of NaCT's sodium and citrate binding, and their coupling.

Below are a few minor comments that require only revision of figures and text.

1-2. Density for each helix should be shown individually with the structural model. At the very least, densities and structural models should be shown for key structural elements such as helices 4a, 4b, 5a, 5b, 9a, 9b, 10a and 10b, and loops such as HPin, HPout, L5ab and L10ab.

As suggested by Reviewer #1, we have now included densities of these helices for both the NaCT- citrate map (Extended Data Fig. 5e) and the NaCT-PF2 map (Extended Data Fig. 7g).

1-3. Citrate densities should be shown in a similar way as these of Na ions or PF2: densities of residues that form the binding site and densities of citrate should be shown simultaneously at the same sigma level.

As suggested, we have now included figures showing the binding site residues, Na^+, and citrate (Extended Data Fig. 6a) or PF2 (Extended Data Figs. 8c&d) at the same sigma level.

1-4. The densities of Na ions shown in Figure 2a and b are weak, given that Na is much heavier than C. Can the authors comment on whether the electrostatics of the Na ion binding sites are sufficient to accommodate a cation? In many transporters and ion channels, Na ion binding sites have explicit charges such as glutamate or aspartate.

We agree that the density at the Na1 and Na2 sites are weak. There are several reasons for this. Unlike the covalently-bound carbons, the Na^+ ions have greater mobility than the surrounding residues.

Perhaps more importantly, the sodium sites are likely partially occupied by the

much lighter Li^+ present in the buffer, which was used to stabilize the protein. As previously shown by other investigators, compared with Na^+, Li^+ was able to enhance citrate transport of NaCT at 1% of the sodium concentration (Inoue et al., Biochem J. (2003) 374, 21; Gopal, et al., J Pharmocol exp Therap., (2015) 353, 17). This indicates that the NaCT protein has a higher affinity to Li^+ than Na^+. As Li^+ scatters electrons much more weakly, this mixture of Li^+ and Na^+ likely caused the weaker density observed at these positions.

Despite the apparently weak cation density, the assignment of Na1 and Na2 sites in NaCT is supported by additional evidence from the mammalian SLC13 family of transporters and their bacterial homologs. First, the NaCT binding sites exhibit clear structural homology to those of its bacterial homolog, VcINDY. In the VcINDY X-ray structures the sodium ions are clearly resolved (Mancusso et al., Nature, (2012) 491, 622; Nie et al., Nat Commun (2017) 8, 15009). Furthermore, extensive experiments have previously shown that the binding of Na^+ and substrate in SLC13 transporters is sequential, with Na^+ binding first, followed by substrate (Wright, et al., J Biol Chem (1983) 258, 5456; Yao et al., Amer J Physiol Ren Physiol (2000) 279, F54; Hall et al., J Bacter (2005) 187, 5189; Pajor J Membr Biol (2013) 246, 705; Mulligan, J Gen Physiol (2016) 143, 745). Therefore, the presence of citrate, indicated by the citrate density, necessitates the presence of sodium ions bound at sites Na1 and Na2.

The two Na^+ sites in NaCT are formed by the side chains of Ser, Asn and Thr, as well as the carbonyl oxygens of peptide backbones. No Glu or Asp side chains are involved. Most notably, NaCT's Na1 and Na2 sites are similar to the Na^+ binding sites of its bacterial homolog, VcINDY, which is also a Na^+ and Li^+ driven carboxylate transporter. Similar binding site chemistries have also been observed in the Na^+-dependent transporters for glutamate (GltPH), glucose (vSGLT) and nucleoside (CNT).

As suggested by the reviewer, we calculated the valence of binding sites (Nayal and Cera, J. Mol. Biol. (1996), 256, 228) in both the NaCT-citrate and NaCT-PF2 structures. At each site, the calculated valence is consistent with sodium binding.

These points have been clarified in the revised manuscript.

1-5. Could the authors indicate whether Na ions were included in the calculation when they show the electrostatic surface for the inhibitor binding site i Figure 3c? Given that PF2 has two negative charges while citrate has three, is there any difference in their requirement for Na? I am not asking for more experiments, but do authors know if citrate binding is dependent on Na ions, and whether PF2 binding is less dependent on Na?

The sodium ions were included in the calculations of the electrostatic surface of the PF2 site (Fig. 3c). All available experimental data on the mammalian SLC13 family of transporters, and their bacterial homologs, indicate a sequential binding of Na^+ first, followed by substrate, in a typical "induced-fit" mechanism. This is consistent with the structures of the bacterial homologs, and the NaCT structure here, where

sodium ions coordinate the loops which bind the substrate's carboxylate moieties. However, we do not know if PF2 binding is less dependent on Na^+. Although lacking a third carboxylate group, PF2's high affinity may be explained by its additional contacts with other parts of the protein through the benzene ring and isobutyl group.

1-6. Can the authors show the electrostatic surface of the citrate binding pocket with and without the presence of the two Na ions? This would help readers understand whether Na ions change the electrostatic surface of the binding pocket. Can the authors comment on whether Na ions can bind to and neutralize the carboxylate on citrate?

We have now included calculated electrostatic surfaces for the citrate site with Na1 and Na2 (Extended Data Fig. 6b). Although the sodium ions are not directly involved in the coordination of the carboxylate substrate, we expect they are indeed necessary for the neutralization of the substrate to lower the energy barrier for its translocation across the membrane. In fact, in our recent paper on the bacterial homologs of NaCT, we proposed that such a charge compensation mechanism as a general principle for all SLC13 proteins and their bacterial homologs (Sauer et al. eLife (2020), 9, e61350).

We have not included calculations of the electrostatic surface of the citrate site without the sodium ions. As discussed above, previous biochemical results on SLC13 proteins have shown Na^+ is necessary for citrate binding. This agrees with our NaCT structure, and with previous high-resolution structures of bacterial homologs, where the sodium ions coordinate the substrate binding loops. In the absence of sodium these loops will likely rearrange. Therefore, electrostatic calculations on the current protein structure without the sodium ions would be non-physiological.

These points have been clarified in the revised manuscript.

1-7. The structural model for the tert-butyl group on PF2 seems odd (Figure 3c and Extended Figure 7f. The central carbon does not seem to be a tetrahedron.

We greatly appreciate the reviewer for pointing this out. We have re-refined the NaCT-PF2 model using quantum-mechanical restraints for the PF2 molecule. This has significantly improved the molecule's fit to the density. As a result, the relevant figures (Figs. 3b-d, 4a; Extended Data Figs, 8e&f) have been updated.

Referee #2 (Remarks to the Author):

In their manuscript the authors describe the architecture of the human citrate transporter SLC13A5 (NaCT), the structural basis for substrate recognition and its inhibition by the selective inhibitor PF2. The study is well performed and provides insight into the functional

mechanism and pharmacology of an important human membrane transport system. Due to the preferred orientation of particles, the structure determination process was challenging. However, the authors overcame this obstacle by data collection at different tilt angles, which resulted in maps of high quality. In light of the bio-medical importance of the investigated protein and the general conclusiveness of results I regard the study as candidate for publication in Nature.

I have several comments the authors should address:

2-1. ??? In light of the data anisotropy and the unusual decay of the Fourier shell correlation shown in Extended Data Fig. 4 f,g, the drop of the correlation coefficient below the 0.143 threshold is a poor indicator for the effective resolution of the cryo-EM density. The authors have acknowledged this by truncating the resolution to 3.00 ??. As even this resolution might be overestimated (as evidenced in the density shown in Fig. 1b which either shows features of anisotropy or over-refinement at its periphery), I suggest that the authors better document the quality of the map in different regions of the protein. This could be done by plotting the local resolution on the map shown in Fig. 1b and by showing density in different regions of the protein including the substrate binding site.

In order to address the data anisotropy, which Reviewer 1 also brought up, and the question of the dynamic mask threshold (below), we have further refined both maps and validated their resolution using two orthogonal methods. The new NaCT-citrate map has a resolution of 3.04 Å by cryoSPARC's corrected FSC (0.143 threshold). Notably, a map calculated from the same particle stack when reconstructed in cisTEM, which does not use masking when calculating resolution, has a resolution of 2.94 Å. This strategy using both masked and unmasked algorithms to calculate map resolution is the current state-of-the-art techniques for processing anisotropic data, as developed in Yifan Cheng's lab (Dang, et al., Nature, (2017) 552, 426; Billestolle, et al., Nature (2020) 586, 807). In the map, the density for Na^{+}s and substrate are present. The PF2 map was reprocessed using the same methodology. Notably, these new maps do not appear over-sharpened after B-factor sharpening, and therefore sharpening and model refinement have been completed without truncating map resolution.

The new FSC and directional FSC curves are now shown in Extended Data Figs. 5a&b. As suggested, we have also included local resolution maps (Extended Data Figs. 5d, 7f) and exemplar densities from different regions of the protein (Extended Data Figs. 5e, 7g).

2-2. ??? In Extended Data Fig.4 f and 6d, the FSC curve for the corrected masked map shows a dip around 4.5A resolution. This can happen during data processing in cryoSPARC as dynamic masking is applied during refinement. As a control, I suggest to either provide

a static mask manually (e.g. after being created in Chimera) or to alternatively lower the threshold for dynamic masking. It would be also good to have an inset with a final mask provided to make sure that no part of the protein density is located outside of the mask.

Based on Reviewer 2's suggestion, we have systematically tested various lower dynamic mask thresholds for both maps, selecting for each the threshold which yielded the smallest dip in the corrected masked map (Extended Fig. 5c). We have therefore updated the corresponding FSC curves and relevant figures (Figs. 2a-c, Extended Data Figs. 5a&b, 6a). We have also included the mask used (Extended Data Fig. 5c).

2-3. ??? The evidence for observing bound Na+ ions in Fig. 2a and b is weak.

This point was raised by Reviewer #1 (point 1-4), and was addressed in the revised version as described above.

2-4. ??? I would be helpful to provide a better representation of the substrate binding site and the surrounding density shown in Fig. 2c. Although the argument that the density of citrate might be compromised by the poor scattering properties of carboxylate groups is credible, it would still be interesting to provide a better view of the region. Similarly, the panels showing the density and interaction mode of the inhibitor could be improved.

The point on the citrate binding site was raised by Reviewer #1 (Point 1-1), and was addressed in the revised version as detailed above. In addition, the parts in the PF2 site are labeled in greater detail to improve the clarity (Fig. 4b).

2-5. ??? Does the inhibitor lock the transporter in its inward-facing conformation? Due to the negative charge I expect the inhibitor not to be membrane permeable. Is it known how it would access its binding site?

Indeed, PF2 locks the transporter in its inward-facing conformation. Huard et al. have previously shown by transport activity measurements that PF2 is imported by NaCT and inhibits the transporter from the cytosolic side (Huard et al., Sci. Rep. (2016), 5, 17391). This is completely in agreement with our NaCT-PF2 structure.

Minor:

2-6. ??? Why was the purification carried out in the presence of Li+? Is Li+ expected to occupy Na+ binding sites even if its concentration is 10-times lower than that of Na+?

The presence of Li^+ greatly increased NaCT's *in vitro* stability, and was therefore essential to its purification.

In vivo, a low concentration of lithium is known to stimulate Na^+-driven citrate transport of NaCT. While in the presence of 140 mM Na^+, the human NaCT protein's citrate uptake is stimulated by as little as 1 mM Li^+ (Inoue et al., Biochem J. (2003) 374, 21; Gopal, et al., J Pharmocol exp Therap., (2015) 353, 17). We therefore expect a significant amount of lithium bound to NaCT under our experimental conditions, likely competing for cation binding sites with sodium as noted above.

We have clarified and emphasized these points in the revised text (Page 9, Paragraph 3).

2-7. ??? What is the net-charge of citrate under physiological conditions?

??? I believe citrate transport is electrogenic, is the charge per transport cycle known?

Citrate transport by NaCT is indeed electrogenic. Under physiological conditions the net charge of citrate being transported is -3, and 4 Na^+ ions are co-transported per cycle (Inoue et al., J Biol Chem, (2002) 277, 39469; Inoue, Biochem J, (2004) 378, 949). However, as noted above, we cannot clearly identify the Na3 and Na4 sites in our current NaCT maps, and therefore do not speculate on their location in the current manuscript.

2-8. ??? It is not clear to me what the mesh in Fig. 3b exactly shows, how the coulombic potential was calculated and at which level the mesh is contoured.

The mesh in Fig. 3b is the measured Coulombic potential for the PF2 molecule. Because electron scattering by the specimen is measured in cryo-EM, the resulting density map directly represents the Coulomb potential of the specimen, in a manner similar to X-ray crystallography where the resulting density map represents the electron density (Marques et al., Curr. Opin. Struct. Biol., (2019), 58, 214).

2-9. ??? How often have experiments in Fig. 1 a been carried out and what was the error between different experiments.

The experiments in Fig. 1a was a screening experiment and were performed four times. These experiments were carried out to search for and optimize

conditions for protein purification, the concentrations of stabilizing agents (Na+, Li+, citrate and PF2) used each time varied slightly.
However, results were within 10 – 15% across different experiments. We have included the raw data for the Fig. 1a experiments as Extended Data Fig 2b to demonstrate the robustness of these results.

Referee #3 (Remarks to the Author):

In this study Sauer et al. determined cryo-electron microscopy structures of human NaCT in complex with citrate and with an inhibitor in order to better understand the structural basis of substrate binding and inhibition mechanism. These structures reveal how NaCT recognizes citrate, how the inhibitor arrests the protein???s transport cycle and why it selectively inhibits NaCT over two homologous dicarboxylate transporters. From these results the authors conclude that the NaCT structures provide a framework for understanding how various mutations abolish NaCT???s transport activity and thereby cause SLC13A5 epilepsy in newborns.

Comments

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3-1. My only suggestion would be to discuss how alterations in citrate transport in the CNS might lead to epilepsy and how reductions in the NaCT transporter might result in alterations in energy metabolism and serve as a target for anti-obesity drugs. This would increase the appeal of this article to non- structural biologists.

While over 40 mutations in the NaCT gene are known to cause SLC13A5-Epilopsy, the pathogenesis of the disease at the level of cell physiology, or greater, is unclear and currently under examination by the field. The current hypothesis in the field is that lower levels of citrate in neurons leads to reduced energy supply and altered neurotransmitter synthesis, both of which are known to cause other types of epilepsy. This point has now been added in the discussion with cited references.

For the optimization of PF2 as a potential anti-obesity drug, we have now made more specific suggestions in the discussion section on how to improve its affinity and selectivity for NaCT.

Reviewer Reports on the First Revision:

Referees' comments:

Referee #1 (Remarks to the Author):

The authors addressed all the questions.

Referee #2 (Remarks to the Author):

The authors have addressed the comments of the reviewers in a satisfactory manner. The comments regarding the Na+ binding sites and transport coupling are sensible. The sodium to citrate stoichiometry was previously investigated in functional studies. Since the substrate binds to a well-defined site in the center of a mobile transport domain, there is no reason to assume that more than a single citrate molecule is transported per subunit and transport cycle. Moreover, the positive net charge of transport and the assumption of a trivalent anionic state of citrate mandates the cotransport of at least four Na+ ions. Although Na+ ions cannot be identified unambiguously in cryo-EM density at the described resolution, the presence of residual density at equivalent locations as identified in the prokaryotic homologue VcINDY makes the assignment credible. It is also reasonable not to speculate on the location of the two remaining Na+ binding sites in absence of conclusive data.
The statistics are generally well-described, although the type of the error displayed in the bars in Fig. 4b and extended Data Figs. 2a, e and 8h and the number of repeats in Extended Data Fig. 2a, e should be defined.

I have few minor comments, which do not require re-review:
In case PF2 enters the cell via NaCT and stabilizes the inward-facing conformation, I wonder whether it could be considered as substrate with low-off-rate?
Page 6 line 1: … all stabilized solubilized protein.
Page 6 Structure determination of NaCT in complex with citrate, check last sentence: (This allowed us…)

Author Rebuttals to First Revision:

We are grateful to the reviewers for their time and effort in reading the manuscript and providing insightful comments. Following their comments and suggestions, we have revised the text and remade many of the figures. Our point-to-point response to their comments is listed below. To make the response clearer, we have taken the liberty of labeling their comments by numbers as, 2- 1, 2-2, … etc.

Referee #2 (Remarks to the Author):

The authors have addressed the comments of the reviewers in a satisfactory manner. The comments regarding the Na+ binding sites and transport coupling are sensible. The sodium to citrate stoichiometry was previously investigated in functional studies. Since the substrate binds to a well- defined site in the center of a mobile transport domain, there is no reason to assume that more than a single citrate molecule is transported per subunit and transport cycle. Moreover, the positive net charge of transport and the assumption of a trivalent anionic state of citrate mandates the cotransport of at least four Na+ ions. Although Na+ ions cannot be identified unambiguously in cryo-EM density at the described resolution, the presence of residual density at equivalent locations as identified in the prokaryotic homologue

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As suggested by reviewer #2, we have added the type of error displayed and the number of replicates to Fig. 4b, and Extended Data Figs. 2a, 2e, and 8h.

2-2. In case PF2 enters the cell via NaCT and stabilizes the inward-facing conformation, I wonder whether it could be considered as substrate with low-off-rate?

We agree with the reviewer that PF2 can be considered as a substrate with a low off-rate on the cytosolic side. While the kinetics of PF2 binding and inhibition is very interesting question, to validate this particular hypothesis will however require a *unidirectional* reconstitution system that can measure the off-rates of the cytosolic and extracellular sides separately. In the interest of scope we have not explored this in our current work.

2-3. Page 6 line 1: ... all stabilized solubilized protein.

We are grateful for the reviewer highlighting this typo. We have corrected it in the finalized manuscript.

2-4. Page 6 Structure determination of NaCT in complex with citrate, check last sentence: (This allowed

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