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SERCA mutant E309Q binds two Ca2+ ions but adopts a catalytic incompetent conformation

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 08 August 2013

Thank you for submitting your manuscript for consideration by the EMBO Journal and my apologies for the delay in communicating our decision to you. Your study has now been seen by three referees whose comments are shown below.

As you will see, the referees all highlight the quality and importance of your findings, although they also raise a number of issues - mainly related to additional explanations and manuscript rephrasing - that you will have to address in order for them to support the publication of a revised manuscript. However, while the additional suggestion made by ref #3 to include data on headpiece dynamics for the E309Q mutant would clearly add further strength to the manuscript, this does not represent an absolute requirement from our side.

Given the referees' positive recommendations, I would thus invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance or rejection of your manuscript will therefore depend on the completeness of your responses in this revised version.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as

soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we may be able to grant an extension.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1

The manuscript describes interesting and important findings related to the role of the invariant residue E309 in SERCA Ca2+-ATPase in the coupling of calcium binding to conformational events in the cytosol leading to catalytic phosphorylation. The functional data are very clear and convincing, and the structure provides important details rationalizing the functional behavior of the E309Q mutant. Overall, this is a worthwhile work that makes a new mechanistic contribution to the field

Two aspects of the study need to be improved
1. It should be stated more clearly (and supported by the available experimental evidence) whether the "flexible" position of the A-domain in the structure is an inherent functional property of the E309Q mutant (i.e. the mutation prevents the A-domain from accepting a fixed position) or a reflection of an insufficient quality of the purified recombinant protein. The authors may address this issue by commenting on whether earlier the same purification/reconstitution procedure was used for the recombinant (non-native) wt SERCA that permitted visualization of the A-domain

2. The disordered nature of the nucleotide-binding pocket and the electron density that does not coincide with AMPPCP need more explanation. The authors should explain how they reconcile the disordered nucleotide-binding pocket and the lack of the fixed position for the adenine moiety with nearly identical (for the wt and the mutant) nucleotide binding curves in the absence of Ca as well as nearly identical TNP-8N3-ATP labeling. Is the "disordered nature of the pocket" relevant to mutant function or it a crystallographic imperfection?

3. Related: since the electron density (phosphate moiety?) appears to be positioned in the immediate vicinity of D351, the reason for the loss of phosphorylation should be explained more clearly, especially given apparent saturation of mutant with ATP

Minor issues

1. The authors are advised to carefully edit the manuscript. Although the text, overall , is concisely and clearly written, it contains a number of grammatical imperfections (omitted articles, missed words, etc) that should be corrected. For example (and more examples can be found): "... 3.5 A resolution based on recombinant enzyme expressed..." - please insert "the" before "recombinant" Later in the sentence ".. strongly reduced compared to wild-type.." change to "strongly reduced when compared with the wild-type..."; on page 6 in the sentence "By structural analysis, we found that...." delete "in" before "the transmembrane domain"..

2. "The core maintains a confluent mass during all the intermediary states of the ATPase cycle that, via this direct connection, may enable the cooperatively interacting Ca2+ ions to fine-tune the phosphorylation site for reaction with ATP" - I do not understand this sentence., it is too complex. What is a "confluent mass"? What does "fine-tune" mean exactly? Please re-phrase using simpler and more specific factual expressions.

3. Similarly:
"...a dysfunctional response to binding at the Ca2+ site II, centred on Glu309 as a critical mediator" - What does this mean?

Referee #2

The manuscript presents the 3.5Å structure of SERCA1a E309Q mutant with bound Ca2+, which is different from wild-type Ca2E1 structure. Although the overall structure of E309Q is not clear, probably due to the flexible nature of A-domain, crystal shape and lattice parameters further supported different configuration of catalytic domains in E309Q. A-domain is roughly assigned in the electron density map for structural comparison with other states. Less-biased average kicked map for Ca2+ binding sites (siteI, II) indicated that both sites are occupied in the E309Q crystal, contrary to the previously reported biochemical analysis.
Authors performed biochemical analysis, revealing that phosphorylation reaction by ATP occurs in E309Q under the higher temperature condition (25ºC). Nucleotide and Ca2+ binding to E309Q mutant were also confirmed by photolabelling experiment and equilibrium 45Ca2+ binding measurement. Considering the inactive fraction of purified E309Q enzyme, it is indicated that two Ca2+ ions bind to the E309Q mutant in a non-cooperative manner.
These structural and biochemical analysis together present the important role of Glu309 in the signal transmission between Ca2+ binding and the catalytic domain that are separated by 50Å. Structural aspects in the checkpoints for phosphorylation reaction, discussed in this manuscript, are of scientifically importance and gives valuable insight into the mechanism in these ATPases, whereas structural and biochemical evidence for two Ca2+ binding in E309Q mutant might be rather weak. In case of the moderate resolution structure, conclusion should be careful. Taken together, the manuscript is acceptable in EMBO J., if the authors properly address the following concerns.

Major points

1. For clarification of the N-domain configuration in the current structure, electron density map for N-domain should be also shown, since the map in Fig.4A is only limited to the nucleotide binding site.

2. Electron density map shown in Fig.3A seems fitting too much to the amino acid residues for its moderate resolution, and R free and work values are too apart (R work/free=20.6/28.6 %), suggesting that the structure is over-refined. This should be explained.

3. Procedures in refinement should be described in Materials and methods. Was the refinement performed without any ligands? Either average kicked map or composite omit map may not remove model bias completely. Refinement cycle should be done without any ligands to discuss about these binding.

4. How pKa values were calculated? Is the bound Ca2+ taken account in the calculation? Is pKa value for Glu58 side chain different for alternative configurations of Gln309? Program used for pKa calculation should be also described in the method section. Even though the calculating process is proper, discussion on hydrogen bonding (ex. page 4, "Glu58 is more likely protonated at neutral pH in E309Q...") might be too speculative for this resolution.

5. Glu309 is involved in Ca2+ binding at site II. Why did E309Q mutation diminish the cooperative binding manner of Ca2+ to SERCA1a, while Glu800 which is shared between these sites is still remaining?

Minor points

1. Programs used for molecular replacement should be described in Materials and methods.
2. Biochemical evidence for the stoichiometry in the bound Ca2+ is based on the roughly estimation of the inactive enzyme ratio. Therefore expression in Discussion should be weakened (ex. Page6, "our 45Ca2+ equilibrium binding measurements confirm that the mutant is able to bind two Ca2+...").
3. Fig.3C: too much speculation in hydrogen bonding patterns and orientation of side chains should be avoided for this moderate resolution structure.

Referee #3

General summary and significance of the study

Glu309 has been considered a possible gating residue in the binding of calcium to SERCA. As a gating residue, Glu309 might play a major role in calcium activation of the enzyme, with effects ranging from ATP coordination and hydrolysis to calcium binding and cooperativity. Previous studies of a Glu309 to Gln mutant abolished calcium transport activity by SERCA. The mutant was unable to undergo calcium-activated phosphorylation from ATP presumably due to the lack of calcium binding at site II (the mutant bound calcium at site I). Furthermore, protonation and interactions involving Glu309 may be important in coordinating the cytoplasmic domains of SERCA, with effects on other transport states such as the dephosphorylation of E2P.

To address the role of this residue, Clausen et al have solved the structure of an E309Q mutant of SERCA at moderate resolution (3.5 Å). The most interesting finding is that the crystal structure of E309Q appears to contain two bound calcium ions. Biochemical analyses reveal micromolar calcium affinity, but cooperativity in calcium binding is lost. The phosphorylation of the mutant from ATP is greatly reduced, and there appears to be a defect in dephosphorylation of E2P under the conditions studied. A mechanistic framework is provided for how calcium binding activates SERCA - the hydrogen bonding pattern and charge neutralization around Glu309 allow for proper positioning of the cytoplasmic domains for ATP utilization.

Overall the manuscript is well written, easily readable and the figures are informative. The findings and conclusions offer novel insight into SERCA function.

Major concerns

The presented work hinges on the unique crystallization behavior and structure of the E309Q mutant. Since the structure appeared to contradict prior biochemical data (published by some of these same authors), the authors re-examined the biochemical behavior of this mutant in response to nucleotide and calcium. The authors discuss the uniqueness of the E309Q crystals, yet the significance of this is questionable and there are many potential explanations for differences in crystallizability. Is it possible that the crystallization conditions have captured a state of the enzyme that does not represent its solution structure - i.e. the openness of the structure and the mobile (weak density) for the A domain (the A domain position is also questionable)? Perhaps it would be useful to include a figure showing the crystal packing of the E309Q mutant so that the crystal contacts are apparent. In addition, it is not possible to assess the fitting of the A domain based on the figures presented (Figure 1B has the potential to be very misleading for the casual reader).

Minor concerns

In the title, the word "uncoupled" is used. While this is a correct description, in the P-type ATPase field uncoupling typically refers to ATP hydrolysis in the absence of ion transport. This is not a feature of the E309Q mutant, thus the title may be a bit misleading to the casual reader. In the context of this manuscript, perhaps the authors mean "non-catalytic" or "catalytically incompetent" conformation?

The last sentence of the abstract is unclear - "...a lack of charge neutralization around Glu309." The authors are referring to the E309Q mutant, which has Gln309 not Glu309.

There are a few odd word choices:
"consonant" on page 6; do the authors mean "consistent"
"concord" on page 7; "agree" might be a more appropriate word.

Non-essential

The E309Q mutant appears to promote an E1-like open conformation of SERCA. An interesting question is whether the mutant disrupts headpiece interactions by altering the dynamics, thermal motions of the N, P and A domains. Studying the headpiece dynamics of this mutant in response to various substrates would be of general interest.

1st Revision - authors' response 07 October 2013

Referee #1:

1. It should be stated more clearly (and supported by the available experimental evidence) whether the "flexible" position of the A-domain in the structure is an inherent functional property of the E309Q mutant (i.e. the mutation prevents the A-domain from accepting a fixed position) or a reflection of an insufficient quality of the purified recombinant protein. The authors may address this issue by commenting on whether earlier the same purification/reconstitution procedure was used for the recombinant (non-native) wt SERCA that permitted visualization of the A-domain.

Our response:
We do indeed interpret the increased flexibility of the A-domain of E309Q, compared with that of the wild type, as the cause of the catalytic deficiencies of the mutant: it is a consequence of an inability of the E309Q mutant to form a compact structure of the cytoplasmic A-, P-, and N-domains in the $Ca_2E1{\cdot}ATP$ and $Ca_2E1P{\cdot}ADP$ states, unlike the wild type enzyme, cf. the corresponding $Ca_2E1{\cdot}AMPPCP$ and $Ca_2E1P{\cdot}AMPPN$ wild type structures (Sørensen et al (2004) *Science* 304:1672; Olesen et al (2007) *Nature* 450:1036). We address this in connection with the discussion of the schematic model in Fig. 8 (page 6, last paragraph in column 2). However, we agree with the concern of the referee, that it could be stated more clearly and have done so in our revised manuscript by inserting the following sentence at the end of the first paragraph of the Discussion:

> "The severe inhibition of ATP utilization in E309Q appears to relate to the straight M1 helix and the disordered nature of the A-domain in the E309Q structure, reflecting an increased flexibility of the A-domain. Thus, the formation of a compact structure of the cytoplasmic A-, P-, and N-domains, enclosing the phosphorylation site and shielding it from the bulk solvent, as displayed by the wild type enzyme in the Ca_2E1/Ca_2E1P states (Sørensen et al, 2004; Olesen et al, 2007), is likely impeded in the mutant. This leads to a diminished ability to attain and/or stabilize the catalytic $Ca_2E1{\cdot}P{\cdot}ADP$ transition state, thus lowering the V_{max} for phosphorylation."

We have no reason to believe that the purity or quality of the recombinant E309Q enzyme is inferior to that of the recombinant wild type. The procedures for the expression, purification, and relipidation applied to mutant E309Q prior to crystallization trials were exactly the same as those applied previously to the recombinant wild type (Jidenko et al (2005) *Proc Natl Acad Sci U S A* 102:11687) and to the recombinant mutants D351A and P312A (Marchand et al (2008) *J Biol Chem* 283:14867). For both of these mutants, crystal structures were determined, and the A domain was visible in both of them. Expression yields of E309Q were similar to those obtained with the wild type, and there were no apparent differences in the purity of the E309Q enzyme compared with the recombinant wild type enzyme, as evaluated by gel electrophoresis and gel filtration analysis (Supplementary Fig. S1, compare with Figs. 15.4 and 15.5 in Cardi et al (2010) *Methods Mol Biol* 601:247). We agree with the referee, that the issue regarding purity/quality/yield of the E309Q enzyme relative to that obtained with wild type was not described in sufficient detail, and we have addressed this in our revised manuscript by including the following sentence in the section "Purification and crystallization of mutant E309Q" under Results:

> "The yield and purity of the E309Q protein was similar to that obtained previously with the recombinant wild type protein (Jidenko et al, 2005), as evaluated by gel electrophoresis and gel filtration analysis (compare Supplementary Figures S1A and S1B with figures 15.5 and 15.4, respectively, in Cardi et al (2010))."

2. The disordered nature of the nucleotide-binding pocket and the electron density that does not coincide with AMPPCP need more explanation. The authors should explain how they reconcile the disordered nucleotide-binding pocket and the lack of the fixed position for the adenine moiety with nearly identical (for the wt and the mutant) nucleotide binding curves in the absence of Ca as well as nearly identical TNP-8N3-ATP labeling. Is the "disordered nature of the pocket" relevant to mutant function or is it a crystallographic imperfection?

Our response:
It was not our intention to convey the notion that the nucleotide-binding pocket is disordered, nor that the electron density we observe does not coincide with AMPPCP. The nucleotide-binding pocket of SERCA is *not* disordered, only the adenosine moiety of the nucleotide ligand is. We do observe electron density for what we interpret as the *beta-* and *gamma* phosphates of AMPPCP (see fig. 4A), which was present in the crystallization condition. We state this on p.4, left column (key statements underlined):

> "Another important finding is that the nucleotide ligand in the E309Q structure is not ordered (Fig. 4A). We observe an electron density peak roughly overlapping with the position of the β- and γ-phosphate in the published [Ca_2]*E*1·AMPPCP structures (when superposed on the P-domain), but the adenosine moiety is invisible in the electron density, unlike the binding pocket itself, indicating disorder."

We have furthermore not meant to indicate that the binding results are directly comparable with the observation from the crystal structure with respect to nucleotide binding. This may be most obvious for the binding studies performed in the absence of Ca^{2+}, because the crystals were obtained in the presence of Ca^{2+}, and both Ca^{2+} ions are bound in the crystal structure.
It should furthermore be noted, that the wild type Ca^{2+}-ATPase has a much lower affinity for AMPPCP than for ATP in the presence of Mg^{2+}, which is known to stimulate ATP binding, but inhibits the binding of AMPPCP (Pang and Briggs (1977) *J Biol Chem* 252:3262; Picard *et al* (2005) *J Biol Chem* 280:18745). The high Mg^{2+} concentration prevailing in the crystallization medium might have interfered even more with AMPPCP binding in the mutant than in the wild type. We agree with the referee that this was not clearly explained in the manuscript and have included the information in the section "The structure of the SERCA E309Q mutant" in Results (second-last paragraph).
Note that in the presence of Ca^{2+} the binding of ATP differs between mutant and wild type in a way that accords with the low phosphorylation rate of the mutant, and this is in line with the observed difference between the crystal structures of mutant and wild type enzyme with AMPPCP.

3. Related: since the electron density (phosphate moiety?) appears to be positioned in the immediate vicinity of D351, the reason for the loss of phosphorylation should be explained more clearly, especially given apparent saturation of mutant with ATP.

Our response:
The nearest distance from the D351 carboxyl to the β/γ-phosphates as indicated by the electron density in question is roughly 4 Å, whereas the distance in the catalytically competent Ca_2E1-AMPPCP structure (PDB 1T5S) is about 2.5 Å. Thus, we don't regard the arrangement we see in the E309Q structure as a catalytically active conformation.
We agree with the referee that this was not stated very clearly in the manuscript. The sentence introduced in the Discussion section in response the question 1 of the referee, as well as the sentence introduced into the Results section in response to question 2, should clarify this issue. Furthermore, we have now mentioned the lack of 'site shielding' in E309Q explicitly in the Discussion section of the manuscript.

Minor issues:

1. The authors are advised to carefully edit the manuscript. Although the text, overall, is concisely and clearly written, it contains a number of grammatical imperfections (omitted articles, missed words, etc) that should be corrected. For example (and more examples can be found): "... 3.5 A resolution based on recombinant enzyme expressed ..." - please insert "the" before "recombinant" Later in the sentence ".. strongly reduced compared to wild-type.." change to "strongly reduced when compared with the wild-type..."; on page 6 in the sentence "By structural analysis, we found that...." delete "in" before "the transmembrane domain"..

Our response:
We have carefully revised the grammar of the manuscript, and we think it has improved notably in the new version.

2. "The core maintains a confluent mass during all the intermediary states of the ATPase cycle that, via this direct connection, may enable the cooperatively interacting Ca2+ ions to fine-tune the phosphorylation site for reaction with ATP" - I do not understand this sentence., it is too complex. What is a "confluent mass"? What does "fine-tune" mean exactly? Please re-phrase using simpler and more specific factual expressions.

Our response:
We have rephrased the entire paragraph to make the message clearer (see discussion, page 7, right column).

3. Similarly: "...a dysfunctional response to binding at the Ca2+ site II, centred on Glu309 as a critical mediator" - What does this mean?

Our response:
With the entire paragraph rephrased this sentence should now also be more easily understood. To further clarify/simplify we have rephrased it as follows (see discussion, page 7, right column):

> "Thus, it is likely that the half-open N-domain position seen in E309Q relative to the [Ca_2]E1·AMPPCP state of native SERCA reflects a defective signal transmission along the central core in response to binding at Ca^{2+} site II, with Glu^{309} being involved as a critical mediator of the signalling."

Referee #2:

...structural and biochemical evidence for two Ca2+ binding in E309Q mutant might be rather weak. In case of the moderate resolution structure, conclusion should be careful.

Our response:
The structural evidence for the two Ca^{2+} ions are peaks of ~10 sigma and ~8 sigma in average kicked omit maps and simulated annealing omit maps, respectively, which are bias-reduced maps calculated with a Ca^{2+} free binding site. The peaks are depicted in Fig.3B. We find this structural evidence to be sufficient and fully and most simply explained by the binding of two Ca^{2+} ions. The biochemical evidence for Ca^{2+} stoichiometry is treated below (minor point #2).

Major points:

1. For clarification of the N-domain configuration in the current structure, electron density map for N-domain should be also shown, since the map in Fig.4A is only limited to the nucleotide binding site.

Our response:
We agree that this is an important point, and we have now included the electron density for the entire molecular model in Figure 1B.

2. Electron density map shown in Fig.3A seems fitting too much to the amino acid residues for its moderate resolution, and R free and work values are too apart (R work/free=20.6/28.6 %), suggesting that the structure is over-refined. This should be explained.

Our response:
The referee is right, the electron density maps are indeed good for the resolution. The reason for this is, however, not overrefinement *per se*, but first of all that a very good starting model was available, derived from known SERCA structures determined at higher resolution (e.g. 1T5S at 2.6 Å). Furthermore a very high solvent content of the crystal form leads to a higher optical resolution of the electron density maps. We have validated all critical conclusions with unbiased omit maps (both simulated annealing- or average kicked omit maps) to exclude the risk of model-induced electron density features (see also our answer to comment 3).
We have carried out an automatic optimization procedure (in *PHENIX*) for the restraint weights of bond angles and bond lengths, which reduced Rfree slightly as well as the Rwork-Rfree gap (26.7/22.3%), which is now at a very low value for a 3.5 Å resolution structure with no NCS.

The electron densities have not visibly changed by the updated refinement. Still, we have re-rendered all structural figures and also updated all statistics and mentioned B-factors based on the updated refinement. The changes are all small and do not alter our conclusions or arguments overall.

3. Procedures in refinement should be described in Materials and methods. Was the refinement performed without any ligands? Either average kicked map or composite omit map may not remove model bias completely. Refinement cycle should be done without any ligands to discuss about these binding.

Our response:
We have added the details of the refinement procedure in the Material and methods sections:

> "[…] refinement was performed with *PHENIX* (Adams *et al*, 2010) using rigid body refinement in initial rounds, followed by refinement of atom coordinates, B-factors and TLS motion (five TLS groups: N-domain, P-domain, M1/M2-, M3/M4-, and M5-M10 helix bundles). Even though the exact position of the A-domain could not be deduced from the electron density maps, including it in to the model (derived from a previously solved structure, placed manually to match the electron density cloud and treated as a rigid body) during refinement and omit map calculation, lead to a considerable improvement of the model phases and thereby the resulting maps. For this reason, all electron density maps, except for those in Fig. 1 and 2, were calculated from models including the A-domain. Unbiased difference Fourier maps (simulated annealing omit maps or average kicked omit maps) were calculated with *PHENIX* to reveal the presence or absence of expected ligands or to evaluate the position of side chains"

All simulated annealing or kicked maps used to discuss ligand binding were calculated without ligands. We refer to them as 'omit' maps throughout the manuscript and figure legends.

4. How pKa values were calculated? Is the bound Ca2+ taken account in the calculation? Is pKa value for Glu58 side chain different for alternative configurations of Gln309? Program used for pKa calculation should be also described in the method section. Even though the calculating process is proper, discussion on hydrogen bonding (ex. page 4, "Glu58 is more likely protonated at neutral pH in E309Q...") might be too speculative for this resolution.

Our response:
We thank the referee for pointing out the importance of this issue. pKa values were estimated using the PROPKA server (prokpa.ki.ku.dk), with PROPKA 3.1, taking into account the bound ligands for the calculation. After having re-refined our structural model (see the above response to comment 2), we re-assessed the pKa prediction. Indeed, the estimated pKa value for Glu^{58} is dependent on the modelled side chain conformation of Gln^{309}. In a conformation where Gln^{309} points towards Glu^{58} with the amide nitrogen, the predicted pKa of Glu^{58} increases (from 5.3 to 6.3). Given the flexibility of the Gln^{309} side chain, we interpret this as a putative hydrogen bonding interaction in the mutant, which cannot appear in the wild type, where Glu^{309} is involved in the bidentate coordination of Ca^{2+} in site II. However, we do acknowledge that pKa estimates will be very uncertain and too speculative on the basis of these *in silico* predictions on a low resolution structure, and therefore we have decided to remove the pKa prediction from the manuscript. In any case, they do not change the important conclusions of the study.
Nevertheless, given the improvement of Fig. 3C (see response to minor point 3), which shows structural support for the positions of the discussed side chains, we still find the general discussion of putative hydrogen bonding patterns justified

5. Glu309 is involved in Ca2+ binding at site II. Why did E309Q mutation diminish the cooperative binding manner of Ca2+ to SERCA1a, while Glu800 which is shared between these sites is still remaining?

Our response:
Asp^{800} is likely important for the cooperative nature of the Ca^{2+} binding of the wild type enzyme, given that it contributes to coordination of both Ca^{2+}-sites. In the E309Q structure, a hydrogen bond

is possible between the Gln^{309} amide group and the main-chain carbonyl oxygen of Asp^{800}, an interaction not likely to occur with the wild type Glu^{309}. The loss of Ca^{2+} cooperativity observed with the E309Q mutant may be a consequence of the disturbance by the Gln^{309} side chain of the Asp^{800} positioning between the two Ca^{2+} sites. We have included this possible explanation for the loss of Ca^{2+} cooperativity in the discussion on page 7:

> "Asp^{800} may be instrumental for the cooperative binding pattern of the wild type, because it coordinates both Ca^{2+} ions, whereas for E309Q it may instead engage in a competing hydrogen bonding interaction with Gln^{309} that leads to a loss of cooperativity."

Minor points:

1. Programs used for molecular replacement should be described in Materials and methods.

Our response:
The used programs are now mentioned in the Materials and methods section as follows:

> "[…] and structure determination by molecular replacement was carried out with *PHASER*, using the *PHENIX AutoMR* function (McCoy *et al*, 2007; Adams *et al*, 2010)."

2. Biochemical evidence for the stoichiometry in the bound Ca2+ is based on the roughly estimation of the inactive enzyme ratio. Therefore expression in Discussion should be weakened (ex. Page6, "our 45Ca2+ equilibrium binding measurements confirm that the mutant is able to bind two Ca2+...").

Our response:
The binding stoichiometry is significantly higher than expected for one binding site, even if all the protein molecules were active in binding. To make this more clear to the reader, we have inserted the following in the Results section on biochemical measurement of Ca^{2+} binding:

> "Because one binding site would correspond to 9.1 nmol/mg protein in a 100% pure and active enzyme preparation (the molecular mass is 110 kDa), and there is inactive protein present in significant amounts, the finding of 12.8 ± 0.7 nmol bound Ca^{2+}/mg is in good accordance with a similar binding stoichiometry of two Ca^{2+} per active Ca^{2+}-ATPase for the wild type and the mutant."

Furthermore, we have added an additional reference where the presence of aggregated protein in an inactive form is demonstrated (Andersen *et al* (1986) Characterization of detergent-solubilized sarcoplasmic reticulum Ca^{2+}-ATPase by high-performance liquid chromatography. *Biochemistry* 25:6439). It is clear from this reference that 12-13 nmol/mg must indicate two Ca^{2+} per active protein monomer.

3. Fig.3C: too much speculation in hydrogen bonding patterns and orientation of side chains should be avoided for this moderate resolution structure.

Our response:
We have revised Figure 3C. It now shows omit maps for all side chains that we discuss in the context of possible hydrogen bonding patterns. Given this structural evidence, we find it justified to discuss **possible** hydrogen bonding patterns, and this is everywhere clear from the text. After review of the structure and the maps, we have re-written the part discussing the observation of two distinct side chain conformations of Gln309. Since this side chain is so highly mobile (as demonstrated by blurred map features and very high B-factors compared to the surrounding atoms), we have to assume that it can rather adopt multiple different conformations. Nevertheless, since we know that it must be there, we find it valid to discuss two possible conformations and explore the possibilities for hydrogen bonding networks of the Gln309 side chain with the immediate surroundings.

Referee #3:

Major concerns:

The authors discuss the uniqueness of the E309Q crystals, yet the significance of this is questionable and there are many potential explanations for differences in crystallizability. Is it possible that the crystallization conditions have captured a state of the enzyme that does not represent its solution structure - i.e. the openness of the structure and the mobile (weak density) for the A domain (the A domain position is also questionable)?

Our response:
This is a general question: Do crystal structures represent relevant structures in solution? Generally we say yes - and the simplest model to explain the literature and the data presented in this manuscript will easily include our crystal structure as it stands. If other interactions and a 'closure' between the N-, P- and A-domains were favourable in E309Q, a crystal structure would most likely show such a closed conformation and not the open one we observe.
The fact that we prepared wild type SERCA in the same way as we did for the mutant E309Q and yet did not obtain the same crystal form as for the mutant strongly suggests that the new crystal form is a direct consequence of the E309Q mutation. The crystals have a solvent content of ~62%, thereby being (and this is generally accepted) a close representation of a fully solvated, native protein in the cell. Due to the high solvent content and the weak nature of protein-protein-interactions in the crystal (few hydrogen bonds and small, buried surface areas), a protein crystal is not likely to *induce* a particular conformation, it could at best *favour* a particular conformation which is also highly sampled in solution. As an example the highly flexible A domain is not forced into any given fixed orientation by crystal contacts. To emphasize this point we have now included two supplementary figures depicting the crystal packing pattern in E309Q and its comparison with the wild type (Supplementary Figures S3A and S3B). Notably, of more than 15 different structural conformations of SERCA obtained in our laboratory through the last nine years (including other recombinant SERCA structures), this is the first time we observe a flexible A-domain like this, and the E309Q crystal form is also clearly related to that of the CaE1 state (1SU4) and the [Ca2]E1P-like states (e.g. 1T5S) – all of space group C2 (see also table 1). As we point out in the manuscript the exact position of the A-domain is not determined, which is also why we didn't include coordinates for the A-domain residues in the deposited structure. The position of the bulk electron density for the A-domain, however, clearly demonstrates that the domain "is there" and its position does not overlap with either the 1SU4 or 1T5S structures, as can be seen from Fig. 2.
Regarding the 'open' N-domain: even if we assume that the observed absolute position of the N-domain is determined by crystal contacts, the fact that the A domain is highly mobile and lacks the rotational freedom to make proper contacts to both the N- and the P domain also supports our model.

Perhaps it would be useful to include a figure showing the crystal packing of the E309Q mutant so that the crystal contacts are apparent.

Our response:
We have now included two such figures in the supplementary section of the manuscript (Supplementary Figures S3A and S3B).

In addition, it is not possible to assess the fitting of the A domain based on the figures presented (Figure 1B has the potential to be very misleading for the casual reader).

Our response:

We agree that the mesh shown in Figure 1B was potentially misleading, and we have changed the figure to show electron density instead. However, as stated in the above answer, we think that the density shown in Fig. 2 makes clear that the A-domain in our structure does not overlap with the position of the A-domain in the two superposed structures.

Minor concerns:

In the title, the word "uncoupled" is used. While this is a correct description, in the P-type ATPase field uncoupling typically refers to ATP hydrolysis in the absence of ion transport. This is not a feature of the E309Q mutant, thus the title may be a bit misleading to the casual reader. In the context of this manuscript, perhaps the authors mean "non-catalytic" or "catalytically incompetent" conformation?

Our response:
We agree that the word "uncoupled" can be misunderstood in this context, as it generally holds a different meaning in the P-type ATPase field. We have replaced it with "catalytically incompetent". Similarly, we have replaced the word "uncoupled" in the abstract with the word "non-catalytic".

The last sentence of the abstract is unclear - "...a lack of charge neutralization around Glu309." The authors are referring to the E309Q mutant, which has Gln309 not Glu309.

Our response:
We agree that the sentence was unclear and have corrected this.

There are a few odd word choices:
"consonant" on page 6; do the authors mean "consistent"
"concord" on page 7; "agree" might be a more appropriate word.

Our response:
We agree and have replaced the words "consonant" and "concord" with "consistent" and "agree", respectively, as suggested by the referee.

Non-essential:

The E309Q mutant appears to promote an E1-like open conformation of SERCA. An interesting question is whether the mutant disrupts headpiece interactions by altering the dynamics, thermal motions of the N, P and A domains. Studying the headpiece dynamics of this mutant in response to various substrates would be of general interest.

Our response:
We agree with the referee that it would be both feasible and interesting to study head piece dynamics of E309Q, e.g. *in vitro* by FRET analysis or *in silico* by molecular dynamics simulation. It would, however, be a major undertaking, and out of the scope for the present work, but we thank the referee for the suggestion.

Additional changes in the revised version of the manuscript:

During the revision of the manuscript we became aware of a minor error in relation to the analysis of the time dependence of phosphorylation of E309Q presented in Fig. 5. Hence, we had normalized the level of phosphorylation of E309Q with 5 μM [g-^{32}P]ATP at 25 ºC in 0.1 mM Ca^{2+} and 5 mM Mg^{2+} (triangles pointing upwards in Fig. 5D) to the same level as that obtained under the same conditions but with 25 mM Mg^{2+} (solid triangles pointing downwards in Fig. 5A). In reality, phosphoenzyme levels of E309Q in 5 mM Mg^{2+} conditions are somewhat higher than those obtained in the 25 mM Mg^{2+} conditions. By re-analysis of the data as well as by the performance of additional experiments measuring the relevant phosphoenzyme levels, we have corrected this error and revised the figure accordingly. The changes to the figure have no impact on the conclusions drawn in the manuscript.

Accepted 21 October 2013

Thank you for submitting your revised manuscript for consideration by The EMBO Journal.

It has now been seen by two of the original referees whose comments are shown below. As you will see they both find that all concerns raised have been sufficiently addressed and they both support publication of the revised manuscript. I am therefore happy to inform you that your study has been

accepted for publication pending a few minor editorial changes as outlined below:

-> Please include the pdb code for the E309Q structure in manuscript
-> Please make sure that information on the nature of the error bars as well as the underlying number of replicas (n≥3) is provide for all figures.

The revised manuscript text can be sent to me by email directly and we will then upload it in house.

If you have any questions, please do not hesitate to contact me. Thank you for your contribution to The EMBO Journal and congratulations on this nicely executed work!

REFEREE REPORTS

Referee #1:
This study reports a crystallographic structure and functional characteristics of the SERCA Ca2+-ATPase mutant E309Q. The work is carefully done, and the conclusions are well substantiated by the data. Overall, the study makes a novel and important contribution to mechanistic understanding of the P-type pumps in general and SERCA Ca2+-ATPase in particular. Specifically, the properties of the E309Q mutant strongly point to a disruption of an inter-molecular communication between the membrane and cytosolic portions of the protein, and the structure provides insights into the mechanism behind the loss of this communication. I found the findings intriguing and illuminating. The authors provided clear and convincing answers to the earlier critique; the manuscript has been edited and overall improved. I have no further concerns

Referee #2:
The authors have answered very well to all the points, and the discussion based on week evidence are removed in the revised manuscript, which has resolved my concern. The added supplementary information is also helpful for understanding the crystal packing. The manuscript now presents structural and biochemical data which is sufficient to discuss about Ca2+ binding and flexibility nature of the A-domain in the E309Q mutant. The "possible" hydrogen bonding in Figure 3C should be also allowed with the additional structural evidence. Taken together, the manuscript is now acceptable in EMBO J.

