THE EMBO JOURNAL

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Mechanism of formate-nitrite transporters by dielectric shift of substrate acidity

Marie Wiechert, Eric Beitz

Corresponding author: Eric Beitz, Christian-Albrechts-University of Kiel

Review timeline:

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Editorial Decision:	28 October 2016
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Accepted:	30 January 2017

Editor: Ieva Gailite

Transaction Report:

(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 28 October 2016

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see from the reports, all referees express interest in the proposed FocA channel transport mechanism reported in your manuscript. However, referees #1 and #3 raise several concerns about the proposed transport mechanism, including questions regarding the function of the FocA transporter in the E. coli system. I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers, but particularly focusing on the verification of the functionality of the suggested transport mechanism in E. coli and addressing the questions about the mechanism of formate transport at neutral pH. I should add that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the main concerns raised at this stage,

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://emboj.embopress.org/about#Transparent_Process

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. Please feel free to contact me if you have any further questions. I look forward to your revision.

REFEREE REPORTS

Referee #1:

The manuscript by Wiechert and Beitz describes attempts to uncover the mechanism of a formate transport protein belonging to the comparatively recently discovered FNT superfamily of pentameric anion-specific channels. The mechanism of these proteins is not completely understood, particularly with regard to how the transport of the substrate through an apparently ‚rigid' and hydrophobic pore in each monomer of the pentamer is achieved from an energetic perspective. Understanding this process is the focus of the current study. The topic is important because these proteins are abundant, especially in anaerobic microorganisms and some single-celled eukarya, they quite possibly represent an ancient transporter family, therefore are relevant to our understanding of the evolution of membrane transport proteins, and have potential medical significance as drug targets because a FNT protein has recently been identified in the malarial parasite as a lactate transporter.

The authors provide evidence that in the heterologous yeast system the FocA protein from E. coli, which is a bi-directional transporter for formate/ formic acid in the natural host, appears to import formate into yeast cells coupled to the pmf. Thus, the suggestion is that the mechanism is formate/proton symport and therefore, if correct, these proteins can be classified as pmf-driven secondary transporters for small anions. The evidence for coupled formate/ proton symport into the yeast cells is compelling; however, the proposed mechanism is not completely convincing. Initially, the focus of the study is on a highly conserved lysyl residue (K156) within the hydrophobic pore of the protein and which was originally suggested to be the proton donor. However, the pKa of this is really too high to act as an effective proton donor. Thus, the final conclusion was that a conserved and ‚fixed' water molecule from the bulk solvent, close to the lysyl residue, acts as the proton donor. According to the authors, the proton „will be transferred at a specific point of substrate approximation to the lysine in the hydrophobic vestibule when the substrate acidity has been sufficiently lowered by the dielectric environment'. There are a number of critical points raised by these findings that are listed below and which need to be addressed by the authors.

1. Reading some of the earlier literature on this subject, this reviewer noted that FocA also transports hypophosphite, a chemical analog of formate. If thie proposed mechanism for FocA is correct, how is it possible that hypophosphite with a pKa of approximately 1.0 can be protonated in this environment?

2. FocA also exports formate in the natural host E. coli. How would export work based on this proposed formate/proton symport mechanism? The authors mention in a sentence in the manuscript that there is also a conserved lysyl residue on the cytoplasmic side of the hydrophobic pore, but is this lysine within the pore cavity?

3. If this mechanism is correct, then theoretically, as long as a proton gradient is present, which is the case in all living cells, then formate will continuously enter the cell as long as the concentration is near the Kd for formate of the transporter. This would result in uncoupling of the proton gradient in these cells and due to acidification of the cytoplasm kill the cells. Therefore there must be a gating mechanism to prevent this occurring, or is this reviewer missing something?

4. Yeast cells cannot deal with high concentrations of formate because they never encounter it apart from the very low-levels likely present during certain biosynthetic reactions. Does E. coli continue to take up formate if it cannot be metabolized?

5. If this mechanism is correct, then the authors should demonstrate that it functions in E. coli cells as well as in yeast cells.

Referee #2:

The manuscript by Wiechert and Beitz addresses the question of the mechanism of formate-nitrite transport. Whilst crystal structures of FNTs have been determined the mechanism of transport has remained elusive. The proposal put forward in this study is that protonation of the substrate in the external vesibule of the transporter is required to neutralize the substrate so as to facilitate passage through a constituatively open pore hydrophobic pore. This is a very innovative and provocative proposal. I found the data supporting the proposal compelling and I am sure that the concepts developed in this study will provoke researchers that work on these transporters and many other closely related to re-evaluate their interpretation of data relating to understanding mechanism of transport and ion permeation in general.

Referee #3:

This elegantly written manuscript by Weichert and Beitz explores the mechanism of transport of the formate transporter FocA that belongs to the formate-nitrate transporter family. The authors tackle questions surrounding the gating and substrate selectivity of the transporter. Overall the paper is very clear and concise. The conclusions are well supported by the data and the authors suggest a mechanism that could be applicable to both FNTs and ammonium transporters.

One aspect I think could be made clearer or discussed in more detail is what species is predicted to be transported at pH 6.8 (where electrical currents are observed). Is it the neutral formic acid? The proportion of this would be very low at pH 6.8 which could account for the significantly reduced uptake rate compared to pH 3.8. But then what is producing the current? Or is it the acid anion substrate formate? This would make more sense as to why current is observed as the substrate would carry negative charge. If this second option is the case, can you truly call this protein a H+-coupled transporter that uses anion/proton symport? Or are there two mechanisms at play - at neutral pH, the transporter is H+ independent and can transport the anion form of substrate while at low pH, the transporter is H+-coupled as the substrate itself is protonated thus resulting in electroneutral transport. In other words, the transporter is not strictly coupled to H+ but can switch between two different mechanisms depending on the environment.

Minor comments;
Page 8 - I found the mention of the cytoplasmic facing lysine confusing as it is not addressed again. If this sentence is retained, 'external facing' or something similar should be inserted in line 13 when switching back to talking about Lys156.
Page 9 - toward bottom, can you restate WT figures for direct comparison with E208Q
Page 10 - 1st line, insert "Formate" or "Substrate" before affinity
Page 11 - line 4, refer to Figure 4D (rather than far right panel)
Page 13 - line 5, I think this should be Fig 5C
Methods - Why are two different protonophores used? What is the pH inside yeast cells?

1st Revision - authors' response 20 December 2016

Point-by-point response:

Referee #1:
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The authors provide evidence that in the heterologous yeast system the FocA protein from E. coli, which is a bi-directional transporter for formate/ formic acid in the natural host, appears to import formate into yeast cells coupled to the pmf. Thus, the suggestion is that the mechanism is formate/proton symport and therefore, if correct, these proteins can be classified as pmf-driven secondary transporters for small anions. The evidence for coupled formate/ proton symport into the yeast cells is compelling; however, the proposed mechanism is not completely convincing. Initially, the focus of the study is on a highly conserved lysyl residue (K156) within the hydrophobic pore of the protein and which was originally suggested to be the proton donor. However, the pKa of this is really too high to act as an effective proton donor. Thus, the final conclusion was that a conserved and ‚fixed' water molecule from the bulk solvent, close to the lysyl residue, acts as the proton donor. According to the authors, the proton „will be transferred at a specific point of substrate approximation to the lysine in the hydrophobic vestibule when the substrate acidity has been sufficiently lowered by the dielectric environment'. There are a number of critical points raised by these findings that are listed below and which need to be addressed by the authors.

1. Reading some of the earlier literature on this subject, this reviewer noted that FocA also transports hypophosphite, a chemical analog of formate. If thie proposed mechanism for FocA is correct, how is it possible that hypophosphite with a pKa of approximately 1.0 can be protonated in this environment?

Response: We do not dismiss earlier electrophysiology data on FNT anion conductance. However, our data indicate that anion/proton symport is the major transport mechanism even at pH 6.8. In particular our observation that protonophores reduce the transport rate to the background level suggests that anion conductance as picked up by electrophysiology is due to comparatively small leak currents. In the study on hypophosphite to which the reviewer is referring and which is cited in our manuscript, very high concentrations of 75 mM were used to yield an inhibitory effect, which is in line with a low anion transport rate. It even seems possible that a certain fraction of the added hypophosphite will become protonated during transport via FocA if one considers the remarkably high rate of pH dependent transport of fluoroacetate with a pK_a only one unit higher than that of hypophosphite. Proton co-transport together with the formate substrate is further physiologically favorable, see response to comment 3 below. We have extended the first paragraph of the discussion section to clarify this point raised by the reviewer. Other sections in the manuscript (p7 top, p15 second paragraph) also mention weak anion conductance of FocA.

2. FocA also exports formate in the natural host E. coli. How would export work based on this proposed formate/proton symport mechanism? The authors mention in a sentence in the manuscript that there is also a conserved lysyl residue on the cytoplasmic side of the hydrophobic pore, but is this lysine within the pore cavity?

Response: The crystal structure of FNT3 by Cyczewsky et al. shows that the conserved lysine at the cytoplasmic side is located within the pore cavity and forms a salt bridge with a conserved glutamate, i.e. a symmetrical layout as the periplasmic lysine. The FocA structures are more heterogenous regarding the N-terminal region including the lysine due to technical issues in the crystallization process: one structure was elucidated with the N-terminus partially truncated, one structure showed a wider conformation of the intracellular constriction (“open” conformation), and a third structure revealed different, probably pH dependent conformations of the N-terminal region. Together, in FocA the N-terminus may be more flexible than in other FNTs, which even may have additional functional consequences. The wording of the sentence the referee relates to has been changed to provide more clarity.

3. If this mechanism is correct, then theoretically, as long as a proton gradient is present, which is the case in all living cells, then formate will continuously enter the cell as long as the concentration is near the Kd for formate of the transporter. This would result in uncoupling of the proton gradient in these cells and due to acidification of the cytoplasm kill the cells. Therefore there must be a gating mechanism to prevent this occurring, or is this reviewer missing something?

Response: The gating mechanism proposed by the reviewer to prevent cytoplasmic acidification occurs at the transcriptional level rather than the protein. Bacteria express a tightly regulated formate-sensitive transcription factor leading to FocA formate transporter production only in the presence of formate under anaerobic conditions. FocA is associated with the formate hydrogen lyase

enzyme complex, which immediately metabolizes entering formate, $HCOO^-$, together with a proton, H^+, to carbon dioxide, CO_2, and dihydrogen, H_2. The latter is used for energy generation. This physiological mechanism shows that in fact co-transport of a proton is required to keep the intracellular pH neutral. We have carried out new experiments in *E. coli*, see also comment 5 below, which show the rapid conversion of formate. Further, we added a new reference (Sawers 2005) and text (2. paragraph in "results", and 1. paragraph in "discussion") to elaborate more on the physiological situation and the bacterial metabolism of formate.

4. Yeast cells cannot deal with high concentrations of formate because they never encounter it apart from the very low-levels likely present during certain biosynthetic reactions. Does E. coli continue to take up formate if it cannot be metabolized?

Response: Formate (plus a proton) will be rapidly metabolized to CO_2 and H_2 by the formate hydrogen lyase enzyme complex after entering the bacterial cell, see response 3 above, preventing accumulation of formate (and protons).

5. If this mechanism is correct, then the authors should demonstrate that it functions in E. coli cells as well as in yeast cells.

Response: We are grateful for the suggestion to demonstrate FocA transport and pH dependency in *E. coli* as this experiment provided insight into the physiological situation and formate metabolism. The new data fully confirm our findings obtained in yeast and are added as Fig. 1G.

Referee #2:

The manuscript by Wiechert and Beitz addresses the question of the mechanism of formate-nitrite transport. Whilst crystal structures of FNTs have been determined the mechanism of transport has remained elusive. The proposal put forward in this study is that protonation of the substrate in the external vesibule of the transporter is required to neutralize the substrate so as to facilitate passage through a constituatively open pore hydrophobic pore. This is a very innovative and provocative proposal. I found the data supporting the proposal compelling and I am sure that the concepts developed in this study will provoke researchers that work on these transporters and many other closely related to re-evaluate their interpretation of data relating to understanding mechanism of transport and ion permeation in general.

Response: We greatly appreciate the highly positive opinion of this referee.

Referee #3:

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Response: see response to comment 1 by reviewer 1 above.

Minor comments;
Page 8 - I found the mention of the cytoplasmic facing lysine confusing as it is not addressed again. If this sentence is retained, 'external facing' or something similar should be inserted in line 13 when switching back to talking about Lys156.

Response: done

Page 9 - toward bottom, can you restate WT figures for direct comparison with E208Q

Response: done

Page 10 - 1st line, insert "Formate" or "Substrate" before affinity

Response: done; “Substrate” added

Page 11 - line 4, refer to Figure 4D (rather than far right panel)

Response: done

Page 13 - line 5, I think this should be Fig 5C

Response: yes, corrected

Methods - Why are two different protonophores used? What is the pH inside yeast cells?

Response: The working range of protonophores is determined by their pK_a. To cover the full pH range as used in the study, two protonophores were required. The explanation and a new reference were added to the methods section.

2nd Editorial Decision 24 January 2017

Thank you for submitting a revised version of your manuscript. It has now been seen by one of the original referees, who finds that all criticisms have been sufficiently addressed and recommend the manuscript for publication. There are just a few remaining editorial issues concerning the text and figures that I need you to address before formal acceptance here.

Please let me know if you have any further questions regarding this final revision. You can use the link below to upload the revised version.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal. I am looking forward to receiving the final version.

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Eric Beitz
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2016-95776

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Three independent experiments with at least triplicate data points each yielded adequate S.E.M. error margins
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	not applicable
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	not applicable
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	not applicable
For animal studies, include a statement about randomization even if no randomization was used.	not applicable
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	not applicable
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Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	not applicable
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Is the variance similar between the groups that are being statistically compared?	not applicable

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	not applicable
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D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	not applicable
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10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	not applicable

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	not applicable
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	not applicable
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	not applicable
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	not applicable
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	not applicable
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	not applicable
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F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	not applicable
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21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	not applicable
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	not applicable

G- Dual use research of concern

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