A conserved heme redox and trafficking pathway for cofactor attachment.

Cynthia Richard-Fogal, Elaine Frawley, Eric Bonner, Huifen Zhu, Brian San Francisco

Corresponding author: Robert G. Kranz, Washington University St. Louis

Review timeline:

<table>
<thead>
<tr>
<th>Event</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Submission date</td>
<td>23 April 2009</td>
</tr>
<tr>
<td>Editorial Decision</td>
<td>08 June 2009</td>
</tr>
<tr>
<td>Revision received</td>
<td>15 June 2009</td>
</tr>
<tr>
<td>Accepted</td>
<td>15 June 2009</td>
</tr>
</tbody>
</table>

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 08 June 2009

Thank you for submitting your manuscript for consideration by the EMBO Journal. I apologise for the delay in getting back to you with a decision but we have still not received the final report from the third referee despite several reminders and telephone calls. Since the other two reviewers seem to be in fair agreement, I would like to provide you with a preliminary decision on your manuscript, in order to save you from any unnecessary further loss of time. As you will see from the comments copied below, both reviewers find your study of the cytochrome C maturation pathway to be novel and interesting and support publication after a number of issues have been addressed which mainly are linked to the presentation of the manuscript.

Given the interest in the study I will consider a revised version for publication, I suggest that you to start to prepare a revised manuscript. However, I will still wait (for the next week until the end of the 15th of June) for the third referee report and therefore I have to stress that this is a preliminary decision and subject to change should the last, missing report bring up serious additional concerns. Once I have received from the third, outstanding referee, I will immediately contact you with his/her comments and finalize the decision. If I have not heard from the referee by this date I will proceed without their report.

Yours sincerely,
Editor
EMBO Journal

REFEEEREE REPORTS
Referee #1 (Remarks to the Author):

The Ccm pathway required for cytochrome c maturation appears to be a very complicated process involving many proteins interacting in different membrane complexes. The mechanism by which heme is covalently attached to apocytochrome is still not understood. In the past years, some of the main breakthroughs in understanding the sequential role and function of Ccm proteins have been obtained through the use of a E. coli Δccm strain grown in aerobic conditions expressing different combination of wt or mutated Ccm proteins followed by the analysis of interactions between proteins, covalent heme binding on CcmE or on a cytochrome c reporter. The data presented by Richard-Fogal and coll. is based on the purification of complexes involved in heme trafficking, analysis of their heme content and of the coordination and redox state of heme iron. The author have carefully check that the modified proteins expressed were functional. They trapped a "pre-adduct" CcmCDE intermediate complex containing a b-type heme in a reduced form (50%). The major unexpected and convincing claim concern the less studied Ccm protein, CcmF which is shown to contain a b-type heme. A conserved His located in a trans membrane domain (new topology studies) was shown to provide an axial ligand for the b-type heme. The authors provide a model involving an early step of heme oxidation and a final iron reduction through CcmF. This work provides important new insights into cytochrome c biogenesis, in particular on the heme redox pathway necessary for holocytochrome c formation. CcmF is proposed to function as a quinol:heme oxidoreductase due to property ubiquinol 1 to reduced CcmF heme in vitro. This is a more speculative proposition in agreement with a possible quinol binding site found in CcmF. However its role has not been investigated in vivo therefore the sentence "CcmF functions as a quinol:heme oxidoreductase" should be modified in the abstract. In the discussion I was expecting some comments concerning the oxidizing environments which would generate ferric heme, and the mechanisms for heme redox control which might have evolved, in particular a comparison with system II.

Comments and questions :

I find that the information provide in figure 1A(although helpful to understand the work presented here) is not exactly a model of the pathway but rather a mixture of model (3 and 5) and description of the complexes purified (1,2,4,5). For the description of purified complexes it would be useful to add on the fig schematic indication of the GST fusions, His tag and mutated proteins all modifications necessary to obtain and purify the complexes (as described at the end of the introduction). I would rather appreciate to find these model complexes in the respective figures 1B,3,4. A separated model of the heme redox and trafficking pathway would be a good support for the discussion.

Concerning the analysis of the pre-adduct and post-adduct intermediate complexes could the authors comment on the possibility of :
- holo CcmE heme oxidation during the purification of CcmCDE post adduct complex ?
- heme b partial oxidation during the purification of CcmCDE pre-adduct complex ?
- GST providing any axial ligand to CcmE when expressing GST-CcmCDE wt and mutant forms?

I suggest to combine fig 5 and 6 (as in fig 2)

The WWD motif in CcmC was also proposed to be involved in interaction with CcmE (Ren 2001) can this proposal be rejected in view of the data presented here ?

Minor points

- check for typing errors
- the reference to Uchida et al 2004 should appear earlier (Tyr 134).
- Supplementary fig1/5/6 : For easier reading check uniform naming of plasmids: pSysl-ΔAB (Sup fig1 and Table 4) = pΔAB(text)=pRGK354. pSystemI/pSysl/pGEXsystemI
I do not find pCompAB in table 4
Legend from figures illustrating purification of different complexes should be (fig 3, 4, compared to Sup fig8 Cr=Crude extract ? What are the % of total fractions loaded ?
Sup fig6 does CE stands for DDM solubilized proteins ?
- Sup table 1 Which organisms were used to determined CcmF amino acids conservation?
Ccm proteins from plant and protist mitochondria should be added.

Referee #2 (Remarks to the Author):

This paper from the Kranz group reports a series of major advances in our knowledge of the complex system I pathway (the CcmABCDEFGH pathway) for cytochrome c assembly in the periplasm of gram-negative bacteria. It provides clear proof of the role of CcmC protein in heme transport, reveals highly stable intermediates in heme transfer from CcmC to CcmE, and confirms the previously proposed role of CcmA / CcmB in releasing heme from CcmC for transfer to CcmF. Perhaps the most surprising result is the discovery that CcmF is itself a cytochrome, a discovery that is well documented by a series of chemical and site-directed mutagenesis experiments. The major challenge that such work faces is that the complexes isolated might be artefacts generated during protein isolation, but such a challenge is easily answered by the range of physical and genetic experiments completed to confirm the preliminary observations. This reviewer was impressed by the careful design and documentation of spectral changes resulting from mutagenesis of histidine residues proposed or known to be ligands to the protein-bound heme groups. This was especially true for the ability to distinguish between the roles of His173 and His261 in CcmF, which established that only the latter is a axial ligand to the iron atom of heme. Note that considerable effort was required to correct the proposed membrane topology of CcmF so that possible ligands to heme can be recognised. Finally, the authors show that CcmF is a quinol oxidase, which in itself is a fascinating discovery. So overall this is an impressive study, well worthy of publication in a top journal. The figures are appropriate (though rather complex) but the supporting material is very helpful - and convincing.

While the quality of the science in this paper is impressive, the authors could make more effort to make their story accessible outside the cytochrome c assembly community. It is inevitable that some abbreviations must be used, but with such a challenging subject, every effort is required to minimise use of unnecessary extra abbreviations. Examples include DDM (dodecylmaltoside) and AP (ammonium persulfate), but there are other examples. I personally dislike an Introduction to include a long final paragraph with a summary of all of the main results in the paper rather than a statement of the aims of the study. There are also the following very trivial points that, if modified, would help the non-specialist reader gain access to an impressive story.

First paragraph of the Result (pity lines are not numbered for easy reference!) Introduce the model system used based upon cytochrome c4? Which "c4" and why?

Second results paragraph - p 8 . Rephrase .....the same split α, "indicating it did not induce one population." This is both ambiguous and unclear.

Throughout: as a purist chemist, I dislike biologists using Na-dithionite; NH4 persulfate (no charges!) Symbols should be used correctly. Also, no hyphen in redox.

Section headed CcmC provides. The start of the third paragraph is the third time it is stated that His60 and His184 are conserved and in the periplasm.

Section headed Trapping. Line 2. Is Lee et al. really the original reference to the discovery that heme is attached to His130 of CcmE? Surely add the original reference. Later: add E to SDS-PAG

Section headed Stoichiometry of the b heme.. Reconstruct the sentence beginning Another method for quantitating heme...

Section headed An axial ligand. The authors need to add the statement that "therefore, H173 is not (or is unlikely to be) a ligand to the heme group. Three lines later, correct spelling of quantitative.
Discussion, second paragraph. Our data indicates - the word data is plural, so the verb should also be plural.

The manuscript by Kranz and coworkers is an excellent paper that describes recent work elucidating the details of the system I cytochrome c maturation (Ccm) pathway in E. coli. Specifically, they have overexpressed and purified for the first time biochemical quantities of some of these proteins, and they have further studied the heme content of the various proteins/complexes as well as ascertained the identity of the key heme ligands. In short, the study is comprehensive and the data presented here are both important and timely. It is an excellent addition to the field, and I suspect that it will be of general interest to people in a number of different fields.

Referee #3 (Remarks to the Author):

Major/General Comments:

1) I am a bit concerned about the putative CcmF/CcmH complex that was purified. While the data are certainly compelling, there is a possibility that the copurification is an artifact of overexpressing the two proteins. It is well known that the overexpression of proteins can sometimes lead to non-physiologically relevant complexes to be observed, and this is especially true when one or more of the proteins is an integral membrane protein with multiple transmembrane sections. This could also explain the varying stoichiometry. Observing copurification of genomically-tagged CcmF and CcmH under their native regulation would have been more compelling. Presumably the authors performed a number of controls to help rule out the possibility of adventitious interactions. These controls should be discussed in more detail.

2) The last part of the Results section entitled "The b heme of CcmF is reduced by quinols, thus it functions as a quinol:heme" is too strongly stated. Strictly speaking the authors have only shown that quinol is capable of reducing the heme in CcmF; they have not demonstrated that this occurs physiologically in the cell. Thus, this particular section remains speculative, and this should be made clear to the reader. The analogous section in the Discussion is better balanced, although it is probably still a bit too strongly stated. (The fact that CcmF contains a region with similarity to known quinone binding sites supports the authors' conclusions. Noting this fact in the Results section in addition to the Discussion section may help the reader.)

3) In a number of instances the authors describe the results for various point mutations. However, it is not clear to me that the authors ever performed experiments to ensure that the structure of the proteins remained unaltered. This is perhaps not important in those instances in which the properties of the mutant enzymes were unchanged relative to wild-type enzymes. In certain instances (such as CcmF(H261A)H), however, the mutants bind less heme. Is this because H261 is crucial to heme binding, or because H261 is important for maintaining proper folding. Simple CD and/or protein-protein interaction studies could easily address this issue.

Other Comments:

1. There are a few instances of awkward sentence structures. These instances include:
   a. Abstract - The eight CcmABCDEFGH proteins traffic heme to CcmF/H? It seems odd to have a protein help traffic heme to itself.
   b. Page 3 - "...the heme cofactor to move through membranes and intracellularly to these proteins requires specific..." This sentence is a bit confusing to read.
   c. Page 4 - "Until now CcmC has not..." This is an awkward sentence because CcmC is part of the complex that is the ABC transporter, and yet the sentence does not make that very obvious.
   d. Page 5 - "...a distinct heme ... never before identified ... is therefore a cytochrome b." This is confusing because the first part of the sentence makes it seem as if the structure of the heme itself is different and that this structure has never been seen before. But if it is a b-type heme, then it is not distinct or unique, and it has certainly been identified before. Presumably you want to say that CcmF was never before identified as being a heme-containing protein. The Abstract has the same potentially misleading language, "CcmF is a cytochrome with a unique heme never realized..." A b-type heme is not unique.

2. Page 3 - "...ferrochelatase, which occurs in the prokaryotic cytoplasm and the mitochondrial matrix." This statement is a bit misleading while the active site of ferrochelatase is certainly in the matrix, the protein itself is attached to the inner mitochondrial membrane. I suggest being more
precise on this statement.
3. Page 3 - "An emerging paradigm on heme trafficking from these components..." This is an overstatement. I suggest cutting this sentence. It simply isn't necessary. I would also suggest starting a new paragraph at the next sentence, where this new paragraph will focus specifically on cytochrome c.
4. In general, the Materials and Methods section seems a bit "thin" to me. I would appreciate additional details and information.
5. Figure 1A - This figure does not indicate the various proteins coming "in and out" of the reaction scheme. Instead the proteins just appear and disappear. Given the space limitations, I'm not sure how this can be accomplished, but I think it would make it easier for the reader if the authors explicitly sketched the various proteins entering and leaving the complex.

Minor Comments:
1. Page 7 - "...a major obstacle...has been to obtain sufficient..." I would argue that it is more accurate to state that the difficulty has not been able to obtain sufficient quantities of enzyme.
2. Page 8 - "...we performed pyridine extraction and spectra..." You can't "perform" spectra. You can, however, obtain, record, or collect spectra.
3. Page 13 - "To further characterize..." Split infinitive.
4. Page 14 - "To unambiguously establish..." This is a split infinitive.
5. Page 15 - "In order to determine..." Delete "In order..."
6. Page 24 - "Heme quantitation and protein purity determination" I suggest changing the heading to "Heme quantification and protein...

7. Oyedotun reference - 562 should be subscripted.

1st Revision - authors' response
15 June 2009

Referee #1 (Remarks to the Author):
The Ccm pathway required for cytochrome c maturation appears to be a very complicated process involving many proteins interacting in different membrane complexes. The mechanism by which heme is covalently attached to apocytochrome is still not understood. In the past years, some of the main breakthroughs in understanding the sequential role and function of Ccm proteins have been obtained through the use of a E. coli Δccm strain grown in aerobic conditions expressing different combination of wt or mutated Ccm proteins followed by the analysis of interactions between proteins, covalent heme binding on CcmE or on a cytochrome c reporter. The data presented by Richard-Fogal and coll. is based on the purification of complexes involved in heme trafficking, analysis of their heme content and of the coordination and redox state of heme iron. The author have carefully check that the modified proteins expressed were functional. They trapped a "pre-adduct" CcmCDE intermediate complex containing a b-type heme in a reduced form (50%). The major unexpected and convincing claim concern the less studied Ccm protein, CcmF which is shown to contain a b-type heme. A conserved His located in a trans membrane domain (new topology studies) was shown to provide an axial ligand for the b-type heme. The authors provide a model involving an early step of heme oxidation and a final iron reduction through CcmF. This work provides important new insights into cytochrome c biogenesis, in particular on the heme redox pathway necessary for holocytochrome c formation. CcmF is proposed to function as a quinol:heme oxidoreductase due to property ubiquinol 1 to reduced CcmF heme in vitro. This is a more speculative proposition in agreement with a possible quinol binding site found in CcmF. However its role has not been investigated in vivo therefore the sentence "CcmF functions as a quinol:heme oxidoreductase" should be modified in the abstract.

Author response: The sentence in the abstract has been modified to read “and in vitro CcmF functions as a quinol:...”

In the discussion I was expecting some comments concerning the oxidizing environments which would generate ferric heme, and the mechanisms for heme redox control which might have evolved, in particular a comparison with system II.
Author response: This is a good point raised by the reviewer and we have added the following to the discussion: “Recently we purified another member of the HHP superfamily, CcsBA, which represents the system II cytochrome c synthetase. We have shown that CcsBA binds heme in its WWD domain (“external heme binding domain”), via analogous, periplasmic histidine axial ligands (Frawley and Kranz, 2009). However, CcsBA binds heme independent of other accessory proteins. Clearly, the CcmC and CcsA proteins show similarities in topology, the WWD domain, and the now proven, functions of external axial histidine ligands. The co-evolution of CcmE with CcmC to form heme binding determinants is unexpected, but certainly reflects the distinct mechanisms operating in the system I pathway.” Also see below Author Response: “Both...”

Comments and questions:

I find that the information provide in figure 1A (although helpful to understand the work presented here) is not exactly a model of the pathway but rather a mixture of model (3 and 5) and description of the complexes purified (1,2,4,5). For the description of purified complexes it would be useful to add on the fig schematic indication of the GST fusions, His tag and mutated proteins all modifications necessary to obtain and purify the complexes (as described at the end of the introduction). I would rather appreciate to find these model complexes in the respective figures 1B,3,4. A separated model of the heme redox and trafficking pathway would be a good support for the discussion.

Author response: This is a good suggestion and we have modified Figure 1A to flow better as a model by showing proteins as they enter and leave the complexes (see reviewer #3 comments) and we refer to the specific purified complexes in the respective figure legends. We have also incorporated the respective tags used for purification of the trapped complexes, keeping in mind that the proteins that contain these tags are all functional, as we note in the manuscript. We have also included a “mini-model” of the protein complexes with each figure that represents protein complex purification.

Concerning the analysis of the pre-adduct and post-adduct intermediate complexes could the authors comment on the possibility of:
- holo CcmE heme oxidation during the purification of CcmCDE post adduct complex?
- heme b partial oxidation during the purification of CcmCDE pre-adduct complex?

Author response: Both of the above questions were addressed by adding “In the case of CcsBA, which is the system II cytochrome c synthetase, heme vinyl groups are directly attached to the CXXCH of apocytochrome c. For the CcmCDE complex, the acceptor is the CcmE His130 residue. As would be predicted, all the heme in purified CcsBA is in the reduced (Fe2+) state, mutations in the flanking histidine ligands are completely non-functional, and these mutant CcsBA proteins have oxidized heme (Frawley and Kranz, 2009).” This result with CcsBA clearly suggests that the purification does not result in oxidation. We then address oxidation in the chemistry underlying adduct formation in the CcmCDE complex.

- GST providing any axial ligand to CcmE when expressing GST-CmcCDE wt and mutant forms?

Author response: If the reviewer is asking whether GST could be providing an axial ligand to the heme of CcmE, and thus participate in binding, the answer is no. Both heme axial ligands come from CcmC and GST fused to other membrane proteins do not purify with heme.

I suggest to combine fig 5 and 6 (as in fig 2)

Author response: We have combined the figures.

The WWD motif in CcmC was also proposed to be involved in interaction with CcmE (Ren 2001) can this proposal be rejected in view of the data presented here?
Author response: Although we find this very unlikely, our data does not address it and we will experimentally address this in future work.

Minors points

- check for typing errors

Author response: The manuscript has been checked for typing (and spelling) errors.

- the reference to Uchida et al 2004 should appear earlier (Tyr 134).

Author response: The reference Uchida et al 2004 has been added to the first mention of Tyr134, the final paragraph of the introduction.

- Supplementary fig1/5/6 : For easier reading check uniform naming of plasmids: pSysl-ΔAB (Sup fig1 and Table 4) = pΔAB(text) = pRGK354.

Author response: A good suggestion by the reviewer and all plasmids noted in the text and in the supplementary figures have been uniformly named.

I do not find pCompAB in table 4

Author response: Plasmid CompAB (pCompccmAB) has been added to supplementary table 4.

- Legend from figures illustrating purification of different complexes should be (fig 3, 4, compared to Sup fig8 Cr=Crude extract ? What are the % of total fractions loaded ?

Author response: We have modified the figure legends of Figure 4 and supplemental figure 8 to include the phrase “compare elution fraction with the load fraction”. The percent of the load and FT fractions are .005%, the wash fractions represent 0.2%, and the elution fractions represent approximately .5%.

Sup fig6 does CE stands for DDM solubilized proteins ?

Author response: A notation that CE is equivalent to DDM solubilized membranes has been added to the Supp Fig 6 legend.

- Sup table 1 Which organisms were used to determined CcmF amino acids conservation?

Ccm proteins from plant and protist mitochondria should be added.

Author response: This is a good idea and we have included a separate sub-heading in Supp table 1 (for CcmF comparison) which includes Oryza sativa, Triticum aestivum, Arabidopsis thaliana, and Tetrahymena thermophila.

Referee #2 (Remarks to the Author):

This paper from the Kranz group reports a series of major advances in our knowledge of the complex system I pathway (the CcmABCDEFGH pathway) for cytochrome c assembly in the periplasm of gram-negative bacteria. It provides clear proof of the role of CcmC protein in heme transport, reveals highly stable intermediates in heme transfer from CcmC to CcmE, and confirms the previously proposed role of CcmA / CcmB in releasing heme from CcmC for transfer to CcmF. Perhaps the most surprising result is the discovery that CcmF is itself a cytochrome, a discovery that is well documented by a series of chemical and site-directed mutagenesis experiments. The major challenge that such work faces is that the complexes isolated might be artefacts generated during protein isolation, but such a challenge is easily answered by the range of physical and genetic experiments completed to confirm the preliminary observations. This reviewer was impressed by the
careful design and documentation of spectral changes resulting from mutagenesis of histidine residues proposed or known to be ligands to the protein-bound heme groups. This was especially true for the ability to distinguish between the roles of His173 and His261 in CcmF, which established that only the latter is an axial ligand to the iron atom of heme. Note that considerable effort was required to correct the proposed membrane topology of CcmF so that possible ligands to heme can be recognized. Finally, the authors show that CcmF is a quinol oxidase, which in itself is a fascinating discovery. So overall this is an impressive study, well worthy of publication in a top journal. The figures are appropriate (though rather complex) but the supporting material is very helpful - and convincing.

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Author response: All instances of DDM and AP, in the main text, have been replaced with dodecylmaltoside and ammonium persulfate, respectively. Where AP appears in Figures we have added “AP is ammonium persulfate” to the appropriate figure legends.

I personally dislike an Introduction to include a long final paragraph with a summary of all of the main results in the paper rather than a statement of the aims of the study.

Author response: We have revised the final paragraph of the introduction to include experimental aims as well as a brief set of results.

There are also the following very trivial points that, if modified, would help the non-specialist reader gain access to an impressive story.

First paragraph of the Result (pity lines are not numbered for easy reference!) Introduce the model system used based upon cytochrome c4? Which “c4” and why?

Author response: A sentence “To test for functionality of GST-tagged CcmC, we used a system that relies on the correct assembly of recombinant holocytochrome c4 (from Bordetella pertussis), as previously described (Feissner, et al. 2006b)” has been added.

Second results paragraph - p 8 . Rephrase …the same α, “indicating it did not induce one population”. This is both ambiguous and unclear.

Author response: The sentence “…indication it did not induce…” has been changed to read “…did not induce one potential population of heme over another (not shown).”

Throughout: as a purist chemist, I dislike biologists using Na-dithionite; NH4 persulfate (no charges!) Symbols should be used correctly. Also, no hyphen in redox.

Author response: All instances of Na-dithionite and NH4-persulfate have been replaced with sodium dithionite and ammonium persulfate, respectively.

Section headed CcmC provides. The start of the third paragraph is the third time it is stated that His60 and His184 are conserved and in the periplasm.

Author response: The first sentence of the third paragraph has been changed to read “We next mutated CcmC His60 and His184 (Fig 2A) to alanines.”

Section headed Trapping. Line 2. Is Lee et al. really the original reference to the discovery that heme is attached to His130 of CcmE? Surely add the original reference. Later: add E to SDS-PAG

Author response: The reference Schulz, 1998 was added. An E was added to SDS-PAG in the sentence “…pre-adduct complex is >95% pure by Coomassie stain of SDS-PAGE...”
Section headed Stoichiometry of the b heme. Reconstruct the sentence beginning Another method for quantitating heme...

Author response: The sentence “Another method for ...” has been changed to “As a complementary approach for quantitating heme we used a method that depends on a heme standard curve and alkali/Triton X-100/methanol extraction (REF). This method does not precipitate...”

Section headed An axial ligand. The authors need to add the statement that "therefore, H173 is not (or is unlikely to be) a ligand to the heme group. Three lines later, correct spelling of quantitative.

Author response: The sentence “...and spectra as wild-type CcmF (Fig 5C)....” has been changed to include “....CcmF (Fig 5C), therefore His173 is not (or is unlikely to be) a ligand to the b-heme.” The misspelling of quantitative was corrected.

Discussion, second paragraph. Our data indicates - the word data is plural, so the verb should also be plural.

Author response: The sentence has been changed to read “Our data indicate...”

Referee #3

Remarks to the Author:

The manuscript by Kranz and coworkers is an excellent paper that describes recent work elucidating the details of the system I cytochrome c maturation (Ccm) pathway in E. coli. Specifically, they have overexpressed and purified for the first time biochemical quantities of some of these proteins, and they have further studied the heme content of the various proteins/complexes as well as ascertained the identity of the key heme ligands. In short, the study is comprehensive and the data presented here are both important and timely. It is an excellent addition to the field, and I suspect that it will be of general interest to people in a number of different fields.

Major/General Comments:

1) I am a bit concerned about the putative CcmF/CcmH complex that was purified. While the data are certainly compelling, there is a possibility that the copurification is an artifact of overexpressing the two proteins. It is well known that the overexpression of proteins can sometimes lead to non-physiologically relevant complexes to be observed, and this is especially true when one or more of the proteins is an integral membrane protein with multiple transmembrane sections. This could also explain the varying stoichiometry. Observing copurification of genomically-tagged CcmF and CcmH under their native regulation would have been more compelling. Presumably the authors performed a number of controls to help rule out the possibility of adventitious interactions. These controls should be discussed in more detail.

Author response: We assume that the reviewer is referring to CcmH co-purifying with CcmF:6xHis. In supplemental Fig 6 we note that CcmH only elutes with a His-tag version of CcmF. Many controls are presented. For example, with the GST-CcmCDEFGH (the pSysI-∆AB plasmid) all ccm proteins (including CcmF and CcmH) are overexpressed, but only GST-CcmC, CcmD, and CcmE co-purify (not CcmF and CcmH). As we state, the copurification of CcmH with CcmF is a minor result, already published by Daldal and colleagues (for R. capsulatus proteins) and we did not want to belabor it.

2) The last part of the Results section entitled "The b heme of CcmF is reduced by quinols, thus it functions as a quinol:heme" is too strongly stated. Strictly speaking the authors have only shown that quinol is capable of reducing the heme in CcmF; they have not demonstrated that this occurs physiologically in the cell. Thus, this particular section remains speculative, and this should be made clear to the reader. The analogous section in the Discussion is better balanced, although it is probably still a bit too strongly stated. (The fact that CcmF contains a region with similarity to known quinone binding sites supports the authors' conclusions. Noting this fact in the Results section in addition to the Discussion section may help the reader.)
Author response: Besides the abstract noted above for reviewer 1, we have changed the heading “The heme of CcmF...” to read “…by quinols, thus it functions in vitro as a...”. We have also added the sentence “We note that CcmF contains a region -(NPF) that is similar to the known quinone binding site of RegB (Swem, 2006) and that the location of this region is in close proximity to the b-heme of CcmF (Figure 5).”

3) In a number of instances the authors describe the results for various point mutations. However, it is not clear to me that the authors ever performed experiments to ensure that the structure of the proteins remained unaltered. This is perhaps not important in those instances in which the properties of the mutant enzymes were unchanged relative to wild-type enzymes. In certain instances (such as CcmF(H261A)H), however, the mutants bind less heme. Is this because H261 is crucial to heme binding, or because H261 is important for maintaining proper folding. Simple CD and/or protein-protein interaction studies could easily address this issue.

Author response: This is a good point raised by the reviewer and we have added the sentence “CcmH still co-purifies with CcmF(H261A)H, indicating that the proper protein-protein interactions are maintained, thus suggesting that CcmF(H261A)H is properly folded.” We also note that even though four-fold less heme binds to CcmF(H261A), the heme that is present is significantly perturbed spectrally, as expected for a heme ligand mutation.

Other Comments:
1. There are a few instances of awkward sentence structures. These instances include:
   a. Abstract - The eight CcmABCDEFGH proteins traffic heme to CcmF/H? It seems odd to have a protein help traffic heme to itself.

   Author response: The sentence "The CcmABCDEFGH proteins traffic heme to CcmF/H? It seems odd to have a protein help traffic heme to itself.

   b. Page 3 - "...the heme cofactor to move through membranes and intracellularly to these proteins requires specific..." This sentence is a bit confusing to read.

   Author response: The sentence "...It is thought that for the the heme cofactor to move through..." has been changed to "Nevertheless, specific mechanisms by which the heme cofactor is delivered to these apoproteins are poorly understood (Hamza, 2006)."

   c. Page 4 - "Until now CcmC has not..." This is an awkward sentence because CcmC is part of the complex that is the ABC transporter, and yet the sentence does not make that very obvious.

   Author response: The sentence “Until now CcmC has not...” has been changed to “Until now CcmC has not been purified, although it has been shown to be part of the CmABCD ABC transporter complex, which is required to release the holoCcmE from CcmC (Figure 1A, 3 and 4*) (Feissner et al., 2006a)."

   d. Page 5 - "...a distinct heme... never before identified... is therefore a cytochrome b." This is confusing because the first part of the sentence makes it seem as if the structure of the heme itself is different and that this structure has never been seen before. But if it is a b-type heme, then it is not distinct or unique, and it has certainly been identified before. Presumably you want to say that CcmF was never before identified as being a heme-containing protein. The Abstract has the same potentially misleading language, "CcmF is a cytochrome with a unique heme never realized..." A b-type heme is not unique.

   Author response: The sentence “…the CcmF/H complex (Fig 1A, 5*) discovering a distinct heme...” has been changed to “…the CcmF/H complex (Figure 1A, 5*) discovering that CcmF contains heme in an equimolar stoichiometry, which has never before been identified.” We have also changed the sentence in the abstract “Surprisingly, CcmF is...” to read “Surprisingly, CcmF is a cytochrome b with a heme never before realized, and in vitro, CcmF functions as a quinol:heme oxidoreductase.”

2. Page 3 - "...ferrochelatase, which occurs in the prokaryotic cytoplasm and the mitochondrial
matrix." This statement is a bit misleading for while the active site of ferrochelatase is certainly in
the matrix, the protein itself is attached to the inner mitochondrial membrane. I suggest being more
precise on this statement.

Author response: The sentence “The last step of heme synthesis...” has been changed to “The last
step of heme synthesis is the insertion of iron (Fe2+) into porphyrin by ferrochelatase, which occurs
in the prokaryotic cytoplasm and the mitochondrial matrix, possibly at the inner membrane (Dailey,
2002; O'Brian and Thony-Meyer, 2002).

3. Page 3 - "An emerging paradigm on heme trafficking from these components..." This is an
overstatement. I suggest cutting this sentence. It simply isn't necessary. I would also suggest starting
a new paragraph at the next sentence, where this new paragraph will focus specifically on
cytochrome c.

Author response: We have removed the sentence "An emerging paradigm on heme..."

4. In general, the Materials and Methods section seems a bit "thin" to me. I would appreciate
additional details and information.

Author response: We added the following sentence to the section Membrane Preparation... “Cells
were harvested at 9,000 x g for 10 min, resuspended in either 1XGST buffer (4.3 mM Na3HPO4,
1.47 mM KH2PO4, 137 mM NaCl, 2.7 mM KCl, pH 7.3) or a modified 1X Talon (Clonetech)
binding buffer (20 mM Tris-HCl, pH 7.9, 100 mM NaCl), treated with 1 mg-1 egg white lysozyme
(Sigma-Aldrich) for 20 min, and sonicated at 50% duty, 80% output on a Branson 250 sonicator.
Membrane proteins were isolated at 207,000 x g for 75 min, resuspended in either 1XGST buffer or
modified 1X Talon binding buffer, and solubilized in 1% dodecylmaltoside as previously described
(Feissner et al., 2006a). We have also added to the sentence in the section Other Methods “Heme
stain and... on un-boiled samples suspended in 6x SDS-PAGE dye without DTT. “

5. Figure 1A - This figure does not indicate the various proteins coming "in and out" of the reaction
scheme. Instead the proteins just appear and disappear. Given the space limitations, I'm not sure how
this can be accomplished, but I think it would make it easier for the reader if the authors explicitly
sketched the various proteins entering and leaving the complex.

Author response: This is a good suggestion to help make Fig. 1A flow more as a model,
similar to what reviewer 1 suggested. We now show CcmAB proteins entering the pathway and
CcmABCD leaving the pathway.

Minor Comments:
1. Page 7 - "...a major obstacle...has been to obtain sufficient..." I would argue that it is more
accurate to state that the difficulty has been not being able to obtain sufficient quantities of enzyme.

Author response: We have changed the sentence to read “...a major obstacle...has been in obtaining
sufficient...”

2. Page 8 - "...we performed pyridine extraction and spectra..." You can't "perform" spectra. You
can, however, obtain, record, or collect spectra.

Author response: The sentence has been changed to read “...we performed pyridine extraction and
obtained spectra...”

3. Page 13 - "To further characterize..." Split infinitive.

Author response: The sentence has been changed to read “To characterize further...”

4. Page 14 - "To unambiguously establish..." This is a split infinitive.

Author response: The sentence has been changed to “To establish unambiguously...”

5. Page 15 - "In order to determine..." Delete "In order..."
Author response: In order has been deleted and the sentence is “To determine....”

6. Page 24 - "Heme quanitation and protein purity determination" I suggest changing the heading to "Heme quantification and protein..."

Author response: The heading has been changed to “Heme quantification and protein....”

7. Oyedotun reference - 562 should be subscripted.

Author response: The -562 of the Oyedotun reference has been subscripted.