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Commentary Response

Further comparison of ubiquinol and cytochrome *c* terminal oxidases

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In the preceeding commentary, Haltia reflects on our view presented in [1] that the ubiquinol and cytochrome *c* terminal oxidases are likely to utilize different electron transfer and proton translocation mechanisms. We would like to take this opportunity to clarify our position on some of the issues that he discusses.

Haltia compares the structure and function of subunit II of the two families of terminal oxidases, the ubiquinol oxidases and the cytochrome *c* oxidases. While structure prediction in general is hardly a mature field, hydrophobicity calculations are relatively accurate for predicting the number and approximate location of transmembrane helices in a membrane protein. However, such predictions are inaccurate at times [2,3] and, moreover, say virtually nothing about the extramembraneous domains. To predict that two polypeptides have a similar structure on the basis of hydrophobicity calculations is pushing the technique beyond the limits of its capabilities. As pointed out in our minireview [1], the Cu_A site and part or all of the residues responsible for the binding of cytochrome *c* are thought to be located in the extra-membraneous domain of subunit II of the cytochrome *c* oxidases. In contrast, due to the extreme hydrophobic nature of the physiological substrate ubiquinol-8, the ubiquinol binding site(s) is (are) expected to exist in the transmembrane region of the ubiquinol oxidase complexes as is analogously observed in the structures of bacterial photosynthetic reaction centers. If a ubiquinol binding domain exists on subunit II of the cytochrome bo_3 complex, as has been suggested by a number of studies [4,5], the substrate oxidizing domains of the two families of terminal oxidases would exist in different three-dimensional locations and protein environments of subunit II. Thus, the subunit II's of the two families of oxidases could have either very different folding patterns to accommodate the different substrates or they could have very similar overall folding patterns for evolutionary reasons. We note that the 10% sequence identity of subunit II of the *Escherichia coli* cytochrome bo_3 complex and the *Paracoccus denitrificans* cytochrome aa_3 complex is well below what Doolittle terms the 'twilight zone' (15–25% sequence identity), and accordingly, these polypeptides are unlikely to be evolutionarily related based on sequence comparison alone [6].

Haltia supports his argument for similar overall folding patterns for subunit II of the two families of oxidases with experiments in which up to six residues in a fragment of the *E. coli* quinol oxidase subunit II were mutated in an attempt to create a Cu_A site [7,8]. It remains to be shown that the peptide conformations with and without the mutated residues are basically the same. Virtually any polypeptide into which cysteines and histidines are introduced will bind copper, most likely in an altered conformation because of the nucleating effect of the metal ions. Van der Oost and coworkers found that mutation of these six residues in the holoenzyme resulted in severely inhibited growth and the mutant enzyme was unstable upon purification [7]. These results indicate that binding of copper to subunit II produces a substantial disruption of the structure of the complex and that significant conformational changes occur upon incorporation of the metal site. Finally, we note that a type I copper site has been introduced into *E. coli* thioredoxin in an environment of very different secondary structure than that found in blue-copper proteins indicating that spectroscopically identical metal sites do not necessarily reflect similar polypeptide folding patterns. In addition, mutated residues are not necessarily utilized as hoped, that is, as ligands to the introduced metal site [9].

We agree with Haltia that the ubiquinol oxidases are the more ancient of the two families of terminal oxidases

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Abbreviations: HQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide; UHDBT, 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole.

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but for a different reason. As pointed out in [1], the cytochrome bo_3 complex combines the electron transfer processes of both the cytochrome bc_1 and cytochrome aa_3 complexes and thus is similar to the cytochrome bc_1/cytochrome *c*/cytochrome aa_3 supercomplexes isolated from *P. denitrificans* [10] and thermophilic bacillus PS3 [11]. We assume that the supercomplexes are the more evolved version of terminal oxidases due to their greater complexity and their greater efficiency at energy transduction. Haltia's argument that the ubiquinol oxidase is the more ancient form because it has a lower copper content and that copper was not generally available until oxygen appeared is difficult to accept. In wild-type *E. coli*, the cytochrome bo_3 complex is expressed under conditions of high oxygen tension (the cytochrome *d* complex is expressed under conditions of low oxygen tension and does not contain copper [12]), which would imply that the cytochrome bo_3 complex evolved well after the appearance of oxygen, and thus copper, in the biosphere.

Haltia also argues that 'the ubiquinol oxidases are likely to have a less efficient, and probably simpler, electron gating mechanism than the cytochrome *c* oxidases, in contrast to the complex mechanism proposed in [1]'. A redox loop as proposed in [1] only requires control of electron transfer since proton flow follows to or from the more accessible proton pool (i.e. aqueous medium). However, a proton pump as found in the cytochrome *c* oxidases requires control of both electron transfer and proton conduction through the enzyme complex as well as efficient coupling between these two processes (redox linkage). Proton conduction is much more difficult to control than electron transfer, and thus, we argue that the mechanism presented in [1] is a simpler proton translocation mechanism than that found in the cytochrome *c* oxidases. This line of reasoning also suggests that the ubiquinol oxidases comprise the more primitive terminal oxidase family.

The midpoint potentials of the redox centers in the cytochrome bo_3 complex are still a matter of debate. In [1], we used recent room temperature data which indicate that the midpoint potentials of cytochrome *b*, cytochrome o_3 and Cu_B are about 60, 220, and 400 mV, respectively [13,14]. Other investigators have found the cytochrome *b* midpoint potential to be about 140 mV [15] or as high as 250 mV [16]. The latter value was obtained by EPR measurements at 77 K, so the observed midpoint potential may be much different than the more physiologically relevant room temperature midpoint potential. The room temperature potential of cytochrome *b* appears to be in the 50–150 mV range which is still > 100 mV less than the potential of the low-spin cytochrome of the cytochrome *c* oxidases. A higher potential for cytochrome *b* than we assumed in [1] does not disprove our model, however, for we know nothing about the ubiquinone/ubisemiquinone and ubisemiquinone/ubiquinol redox couples operative in the cytochrome bo_3 system; that is, we do not know the extent to which the cytochrome bo_3 complex stabilizes ubisemiquinone. Rationalizations of complex functional processes based on midpoint potentials are admittedly quite weak since the important parameters are physiological solution potentials about which we know little. Our use of midpoint potentials in [1] was not intended to prove our model but rather to illustrate an alternative method of interpreting data concerning the function of the cytochrome bo_3 complex.

Electron transfer from cytochrome *b* to cytochrome o_3 in the context of the model presented in [1] is an electron leak which results in a slip in the proton translocation process. Such an observed electron transfer has been observed in CO-flash experiments [17,18] and may result from the non-physiological nature of the fully-reduced cytochrome bo_3 complex in its CO-bound and -unbound forms. More importantly, however, the model presented in [1] requires the presence of a ubiquinone molecule at the Q_B site. Such a bound ubiquinone molecule can have a profound effect on the open electron transfer pathways due to redox as well as conformational interactions. To our knowledge, ubiquinone was not present in the CO-flash experiments mentioned above so these data do not disprove the model in [1]. We note that a model of the full turnover cycle of the cytochrome bo_3 complex may require electron transfer between cytochrome *b* and the binuclear center.

Due to the similarity between the Q-loop of the cytochrome bc_1 complex and that which we propose for the cytochrome bo_3 complex, one can predict the manner in which various ubiquinone analogs will affect the activity of the cytochrome bo_3 complex. For example, 2-heptyl-4-hydroxyquinoline *N*-oxide (HQNO) and 5-*n*-undecyl-6-hydroxy-4,7-dioxobenzothiazole (UHDBT) have been found to inhibit the activity of the cytochrome bo_3 complex with K_i's of 0.8 and 0.3 mM, respectively [19]. These inhibitors are also potent inhibitors of the cytochrome bc_1 complex, and bind at different sites on this enzyme. HQNO inhibits ubiquinone binding to center N [20] whereas UHDBT inhibits ubiquinol binding to center P [21]. Thus, we predict that HQNO inhibits ubiquinone binding to the Q_B site and UHDBT inhibits ubiquinol binding to the Q_A site according to our Q-loop model for the cytochrome bo_3 complex. HQNO has been found to modify the spectroscopic behavior of cytochrome o_3 and inhibit reoxidation of this cytochrome by oxygen [15], results that Haltia terms 'anomalous'. These results could be explained by conformational interactions between the Q_B site and cytochrome o_3 but this explanation is unlikely due to proximity of the Q_B site to cytochrome *b* and the absence of any spectral perturbations to this cytochrome. However, HQNO has been reported to affect both center P and center N at high inhibitor to protein ratios [20], and thus, it is conceivable that HQNO binds to both the Q_A and Q_B sites at the high concentrations

used in [15]. Under these conditions, then, it is feasible that there are conformational interactions between the Q_A site and the high-spin heme but not the low-spin heme.

There obviously exists much that we do not yet understand about the family of terminal ubiquinol oxidases. We have raised some intriguing possibilities that can only be settled by further experimentation, and we hope that this discussion aids in directing such investigations.

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