Roles of cytochromes *c* and *b*₅ in mitochondria and microsomes:

Classical and murburn perspectives

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ABSTRACT: While cytochrome *c* (Cyt. *c*)s is a soluble protein involved in mitochondrial electron transfer (ET) reactions between Complex III and Complex IV, cytochrome *b*₅ (Cyt. *b*₅) is a microsomal membrane protein acting as a redox aide for diverse cytochrome P450s and their unique reductase. We found little conservation in the sequence and surface amino acid residues of Cyt. *c* and *b*₅ proteins among evolutionarily diverse species. Hence, the logic that these proteins mediate ET through affinity binding via specific surface residues is weak. Also, analysis of putative protein-protein interactions in the crystal structures of these proteins and their redox partners did not point to any specific interaction logic. The literature on kinetic and thermodynamic constants of mutants (with altered surface residues) did not provide strong evidence to support the binding-based ET paradigm. Topographically divergent Cyt. *b*₅ from one species has been shown to enhance the activity of CYP450 from another species, implying the involvement of non-specific interactions. These observations downplay the classical protein-protein binding based long range ET mechanism. Further, we provide evidence to show that murburn concept presents better chemico-physical logic for ETs mediated by Cyt. *c* and *b*₅. To explain for the promiscuity of interactions, we conclude that the two proteins act as non-specific/generic redox capacitors, mediating a one-electron redox equilibrium involving diffusible reactive oxygen species (DROS) and ions.

Keywords: Murburn concept; cytochrome *c*; cytochrome *b*₅; electron transfer; protein-protein interactions.

Abbreviations: Cyt. *b*₅R – Cyt. *b*₅ reductase; Cyt. *b*₅ – cytochrome *b*₅; Cyt. *c* – cytochrome *c*; CcO/COX – Cytochrome *c* oxidase; CcP – cytochrome *c* peroxidase; CcR – ubiquinol: cytochrome *c* reductase; CPR – cytochrome P450 reductase; CYP/P450 – cytochrome P450; ET – electron transfer; mOxPhos- mitochondrial oxidative phosphorylation; TMS – transmembrane segment;

INTRODUCTION

Cytochrome *c* (Cyt. *c*), a small (~ 12 KDa protein; $25 \times 25 \times 35$ Å dimensions) is involved in electron transfer from ubiquinol: Cyt. *c* reductase (CcR, Cyt. *bc*₁ complex or complex III) to cytochrome *c* oxidase (COX/CcO or Complex IV). It is found ubiquitously in prokaryotic bacteria, and in mitochondria of algae, fungi, plants and animal cells (Bertini et al., 2006). Cytochrome *b*₅ (Cyt. *b*₅) is a small, hydrophobic, and somewhat acidic cylindrical protein (~16-17 KDa; dimensions of 31×37 Å). It is a modulator of cytochrome P450 (CYP) reactions, connecting electron transfers with CYPs' reductase (cytochrome P450 reductase or CPR) in the reaction milieu of microsomes, wherein, endobiotics as well as xenobiotics (e.g. drugs and pesticides) are metabolized (Schenkman and Jansson, 2003). Cyt. *b*₅ is known to be essential for fatty acid biosynthesis in the microsomes (Oshino et al., 1971). These two proteins contain a single heme moiety as the organic co-factor/prosthetic group in which, iron is the central metal atom. The functional chemistry of these proteins involves change in the redox state of the iron atom between Fe²⁺ and Fe³⁺ and thereby, these proteins can donate or accept electrons (Battistuzzi et al., 2001). Cyt. *c* contains histidine and methionine residues at the two axial positions, respectively, while Cyt. *b*₅ contains two histidine residues at both the axial sites (Battistuzzi et al., 2002).

Cytochromes *c* and *b*₅ are deemed to be specific binding-based electron transfer (ET) agents. These proteins supposedly bind to their redox partners through charge and topography based intermolecular forces which are mediated by specific surface aminoacid residues (Hunte et al., 2002; Nisimoto and Lambeth, 1985). In some cases, even ionic or hydrogen bonds have been reported in the protein-protein interaction interface (Shimada et al., 2017). In eukaryotes, Cyt. *c* has been found to have either donor or acceptor roles with its multiple redox partners; it was shown to bind to neuroglobin (Tiwari et al., 2018), p66^{shc} (Giorgio et al., 2005), CYP450 and apoptosis activating factor-APAF-1 (Hüttemann et al., 2011). The other well-known redox partners are cytochrome *c* peroxidase (CcP), Cyt. *b*₅, Cyt. *c* reductase/Cyt.*bc*₁ complex (*Cbc*₁), cytochrome *c* oxidase (CcO), flavin-dependent sulfhydryl oxidase (Erv1), flavocytochrome *b*₂ (*Fcb*₂) and sulfite oxidase (SOX) (Volkov and van Nuland, 2012). The P450 surface residues which were responsible for binding to Cyt. *b*₅ were found to vary (Bart and Scott, 2017). Cyt. *b*₅ was reported to bind to myoglobin, α -hemoglobin, several CYPs, stearyl co-enzyme A desaturase, Δ^5 & Δ^6 desaturase, plasmalogen synthetase, methylsterol monooxygenase, Δ^7 -Sterol δ^5 -desaturase, 17 α -hydroxylase, CYP11A1, C17,20 lyase, metmyoglobin reductase, methemoglobin reductase and also Cyt. *c*

(Qian et al., 1998; Schenkman and Jansson, 2003). Since these two small proteins have so many important physiological roles such as respiration/energy metabolism, cell survival & apoptosis, fatty acid biosynthesis, steroidogenesis and drug metabolism/detoxification, it is important to study their precise mechanisms of action in order to better explicate their roles in health and disease. Hence, we deemed it necessary to verify if the hypothesis of protein-protein contact-mediated ET in small heme proteins such as Cyt. *c* and *b*₅ held merit in the context of accumulated literature and other aspects of contemporary awareness.

METHODOLOGY

The protein-protein interactions between these cytochromes and their redox partners have been supported using methodologies such as X-ray diffraction, UV-visible spectroscopy, surface plasmon resonance spectroscopy and kinetics assays (Kandel and Lampe, 2014; Salamon and Tollin, 1996). Also, determination of thermodynamics parameters such as association/dissociation constant (König et al., 1980) and comparison of ET rates between native and mutated proteins has been used as a strategy to ratify the role of key residues in facilitating protein-protein associations between Cyt. *c/b*₅ and their redox partners (Hüttemann et al.). Despite considerable steps taken towards unraveling the exact mechanism of ET between Cyt. *c/b*₅ and their redox partners, there are significant discordances in published literature (Kaspera et al., 2011; Porter, 2002). While some reckon that specific surface residues are involved in intermolecular ET (Shimada et al., 2017), others maintain that non-specific charge-based interactions are responsible for the interactions (Mulrooney et al., 2004; Rodgers and Sligar, 1991). For example, the proposed stoichiometry of Cyt. *c*:CcP oscillated back and forth from 1:1 to 2:1 throughout the years since the discovery of CcP in yeast in 1939 (Volkov et al., 2011). It is beyond the scope of this article to address all the literature on interactions of Cyt. *c* and Cyt. *b*₅ with other partner proteins because the field is inundated with innumerable publications which overemphasize the role of protein-protein interactions. On the subject of long distance ET between redox proteins, we had reported that protein-protein binding between flavoprotein reductase (donor) and heme protein (acceptor) are not necessary by demonstrating that ET occurred even when the two proteins were separated by a dialysis membrane (Manoj et al., 2016b). This in turn, was supported by the finding that heme enzymes could mediate ETs with (to and from) their small molecule substrates even without the latter undergoing any binding at the active site (Manoj, 2006; Manoj and Hager, 2008; Manoj et al., 2016b; Manoj et al., 2016c). Also, we recently reported that plastocyanin and ferredoxin (two key ET agents of the Z-scheme of oxygenic

photosynthesis) are not specific binding-based agents, but are generic redox capacitors/redox buffers (Manoj, 2018b; Manoj et al., 2010; Manoj et al., 2019a; Manoj et al., 2016b). These insights justify an exploration of the structure-function correlations of small ET proteins such as Cyt. *c* and Cyt. *b₅*, both of which are seen as non-specific redox capacitors in the murruburn reaction mechanistic purview. Evolutionarily, if protein-protein interactions between two intended partners (say, a and b) were the functional route, it would be a better strategy to enhance the availability and specificity of the proteins a/b and not complicate things by having a third protein (say, c) within the same system. Therefore, we used simple quantitative yardsticks (ET rates, binding constants, etc.) and bioinformatics tools (Rice et al., 2000) to verify the binding-based interaction logic between the proteins (Cyt. *c* and *b₅*) and their redox partners. The structures of proteins retrieved from protein data bank were analyzed using visualization and sequence alignment tools (Pettersen et al., 2004; Rice et al., 2000; Waterhouse et al., 2009).

RESULTS AND DISCUSSION

I. Analysis of Cytochrome *c*

1. Overall conservation of structure of Cyt. *c* and its heme coordination environment

The structure of Cyt. *c* comprises of a single polypeptide chain containing usually 100-104 aminoacid residues in higher organisms; however, in different organisms, it can possess anywhere from 85-135 aminoacid residues (Salemme, 1977). While majority of Cyt. *c* proteins possess five α -helices, some of them contain a small percentage of β -sheets also. A characteristic CXXCH motif is responsible for heme protoporphyrin-IX binding (Allen et al., 2003). Two conserved Cys residues tether the heme edge to the protein and the two axial positions of the coordination sphere of Cyt. *c* are occupied by either H and M or alternatively, 2 H residues. *c*-type cytochromes possess significant structural diversities – for example, Cyt. *c* and *c₂* are all α proteins, while other *c*-type cytochromes such as Cyt. *f*, *c₃*, photosynthetic reaction centre Cyt. *c*, Cyt. *c'* and Cyt. *c₅₆₆* contain other kinds of folds (Barker and Ferguson, 1999). However, heme is tethered via two conserved Cys residues in *c*-type cytochromes in all these proteins and has similar ET roles. Alteration of the coordinating M residue's S-atom with substitutes such as O, N and Se changed the ET/kinetic characteristics, spectroscopic signatures and redox potentials of Cyt. *c* (Battistuzzi et al., 2002). Hence, the coordination sphere of the protein is a vital factor in controlling the overall redox potential of the protein; however, the protein environment is also a contributing factor in the redox potential of Cyt. *c*

which is between +200 and +350 (Battistuzzi et al., 2002; Battistuzzi et al., 2001). While the Fe-N (His residue) bond lengths are unaltered in both reduced and oxidized Cyt. *c*, the Fe-S (Met residue) bonds were found to be significantly longer in ferriCyt. *c* (2.33 Å) than in ferroCyt. *c* (2.29 Å). Apparently, the redox-state-dependent S-Fe bond length alteration was deemed to provide reorganizational energy for fast ET between Cyt. *c* and its partners (Cheng et al., 1999). Other factors which influence the redox potential of Cyt. *c* are - polarity of heme, solvent accessibility, heme propionate solvation and electrostatic interactions within the heme coordination sphere (Zaidi et al., 2014). The heme coordination spheres of ~12 different Cyt. *c* proteins compared in this work are given in Table S1. The M residue's S-atom to heme-Fe bond lengths range from 2.292-2.426 Å, while the H residue N-atom to heme-Fe bond lengths are in the range of 1.913-2.100 Å. From this data, it is apparent that there are significant diversities in both axial ligand atoms'-heme Fe bond lengths. The structure of human Cyt. *c* and its heme coordination sphere is presented in Figure 1. We found very little sequence conservation across species in Cyt. *c* within the evolutionary spectrum- from photosynthetic bacterial Cyt. *c* (*Rhodobacter sphaeroides*, 1DW3) to its rice (1CCR) and human mitochondrial (2N9I/5TY3) counterparts. When taking *Bos taurus* Cyt. *c* (6FF5) as a control, the identity % of these diverse species were found to be remarkably different. An evolutionary tree was constructed to show the evolutionary relationships between the sequences of the chosen proteins (Figure S1). This shows that the overall aminoacid sequence of Cyt. *c* protein is diverse in different organisms. While fish (tuna) and other mammalian (human, horse, monkey and rat) Cyt. *c* have greater identity and similarity % values, *Rhodobacter*, *Leishmania* and *Pichia* Cyt. *c* score very low against the *Bos taurus* control. These details are available in Table 1 and the topology (of select proteins) and sequence comparison are shown in Figures 2 & 3 respectively.

2. Evaluation of key surface residues of Cyt. *c* which are considered necessary for binding to its redox partners

A. Surface residues supposedly involved in Cyt. *c*-CcR complex formation:

Residues C14, C17, H18, G29, P30, G41, N52, W59, Y67, L68, P71, P76, T78, M80 and F82 positions were reported to be highly conserved in various Cyt. *c* proteins across the domains of life (Zaidi et al., 2014). Since CcR and CcO are two very important protein mitochondrial mOxPhos ET complexes with which Cyt. *c* is deemed to form protein-protein interactions, we surveyed literature to find the highly conserved surface residues which are vital in its functionality. Mitochondrial Cyt. *c* proteins possess a high % of K residues (around 16-19 K

out of a total of 104 aminoacids). Several independent experiments had confirmed the important roles of residues K8, 13, 27, 72, 86 and 87 in binding to Cyt. c_1 of the Cyt. bc_1 complex, i.e., CcR/mitochondrial Complex III (Ahmed et al., 1978; König et al., 1980; Sarewicz et al., 2008; Speck et al., 1979). The minor roles of other K residues (73 and 79) have also been acknowledged in the intermolecular ET afforded by them towards electrostatic interactions with the negatively charged surface of CcR. In another report on the crystal structure of a CcR complex with Cyt. c , the Cyt. c_1 heme- Cyt. c heme distance was 17.4 Å and the vinyl groups of the two heme cofactors were only 4.5 Å apart (Lange and Hunte, 2002). In that work, nonpolar interactions with A103, F230, M233 and E235 (in Cyt. c_1) through T12, R13, V28, A81 as well as “potential polar interactions” proffered by K79 and K86 (with A164 and E235 of Cyt. c_1) were shown to be involved in close contact (<4 Å) between Cyt. c_1 and Cyt. c . However, in the same paper, Lange and Hunte (2002) suggested that the stabilization of the interaction between Cyt. c - c_1 and the conserved K residues (79 and 86) contribute only via long-range electrostatic interactions which may aid the proteins in attaining transient states during the process of binding/unbinding (Lange and Hunte, 2002).

In our analyses of the surface residues of 12 different Cyt. c proteins across several species, among 10+ K residues which are located on the surface (except in 1DW3, which has just 4 surface residues), the residues K8, 13, 27, 72, 86 and 87 were found to be conserved in higher organisms’ (esp. mammalian) Cyt. c proteins. These residues were entirely absent in 1DW3 & 4DY9, while only K87 was present in 1CCR. Also, not all of K residues (4-5 out of 6 K residues) were found on the surface of other species’ Cyt. c (eg., 5DFs and 3CX5) (please see Table 1 and Table S2 for more details on all surface residues of Cyt. c present in diverse species). The overall topography of select Cyt. c molecules (Figure 2) and conserved surface residue numbers (as per literature discussed herein) are mapped on Figure 3 to provide a visual representation. The surface topographies of the heme containing (and consequently, the interacting) side of Cyt. c shows that these positively charged residues are not necessarily identical/conserved through the course of evolution. Moreover, the kinetics and thermodynamics data in literature do not ratify protein-protein facilitated intermolecular ET, as given in the pertinent discussion section. As for the other aminoacids (K73 & 79 plus T12, R13, V28, A81), only 2YCC has an R13 residue. T12 is not present in any of the proteins analyzed in this work and, V28 is found on the surface of only 1CCR and 3CYT. A81 is present only on the surface of 1YCC. Another report shows that *Arabidopsis thaliana* (plant) mitochondrial Cyt. c_1 possesses two different binding sites for Cyt. c (Ferguson-Miller et al.,

1976). In that work, apart from the N- and C-terminal regions of the two proteins, residues A13, E15, K16, R19, T20, Q24, Q36, S55, A58, K80, K81, G85, V89, K94 and K95 were found to be in close contact with plant mitochondrial Cyt. *c*₁ and hence, were referred to as “active” contact residues (Moreno-Beltrán et al., 2014). In that work, no passive residues of Cyt. *c* were identified in the plant Cyt. *c*₁-Cyt. *c* complex. However, they discovered that around 47 different residues of Cyt. *c*₁ were “passive” contact residues because of their >50% solvent accessibility. We doubt if 40+ residues of a protein interact with another protein. Moreover, the same study reported the presence of two distinct binding sites for both oxidized and reduced Cyt. *c* based on their ab-initio Brownian dynamics calculations and NMR-driven docking computations; while the first site is a non-productive distal site, another one is a functional/proximal site wherein the heme groups of the two proteins were reckoned to be close enough to each other to allow direct electron transfer (Moreno-Beltrán et al., 2014). The literature is filled with such examples where several different residues have been proposed as surface contact points involved in Cyt. *c* docking to Cyt. *c*₁. This clearly shows that the mechanism of interaction of proteins is as yet unclear. There are clear discrepancies in crystal structures of protein-protein complexes of Cyt.*c* with its partners and therefore, some have attempted to identify the specific interactions using molecular dynamics and protein-protein docking studies (Roberts and Pique, 1999). Given these observations, we hold that there is no strict conservation of these surface residues.

B. Surface residues supposedly involved in Cyt. *c*-CcO complex formation:

CcO/Complex IV is also known to possess surface residues which have high affinity binding for Cyt. *c*. The following specific van der Waal’s interactions between Cyt. *c*-CcO residue pairs were reported: A83-H102, I81-Q103, F82-Q103, heme edge-W104, I81-W104, F82-Y105, Q12-Y121, K13-Y121, A83-E157, A83-D158, K72-L136, P76-L136, G77-L136, K87-I66 and K86-S67 (Roberts and Pique, 1999). A Japanese group reported that the interaction between Cyt. *c* and CcO was “a new class of protein-protein interaction” which they termed as “soft and specific” (Shimada et al., 2017). Another study showed that the contact surface area of subunit II of CcO is much smaller than that of other ET partners of Cyt. *c* and hence, there are much fewer interactions between the subunit of CcO containing CuA, the first redox cofactor in the path of ET from Cyt. *c* to CcO; the residues which were found to be important for protein-protein interaction were – K8, Q12, K13 and K87 (Shimada et al., 2017). In the interaction of CcO with Cyt. *c*, 94% of the surface-surface contacts of Cyt. *c* were found to be mainly with subunit II of CcO, while other minor contacts were observed with VIb and

subunit I. Intermolecular interactions between CcO and Cyt. *c* were found to be a) salt bridges between K8 (Cyt. *c*) and D139 (CcO subunit II) as well as K87 (Cyt. *c*) -D119 (CcO subunit II), b) hydrogen bonds between Cyt. *c*-CcO subunit II –i) Q12-D139, ii) K13-Y105 and Y121. While these interactions were observed near the open end of Cyt. *c* possessing the heme moiety, other interacting surface residues of Cyt. *c* were deciphered through chemical modifications (K8, K13 and K87) and site directed mutagenesis to produce mutants K13L, K86L and K87L which possessed significantly lower ET rates than WT Cyt. *c*.

We analyzed the horse Cyt. *c*- *Bos taurus* CcO complex (PDB ID: 5IY5) and found that the distance between heme iron of Cyt. *c* and Cu_A of CcO was 22.3 Å to just the first of the copper atoms present therein. Shimada et al. also showed that the distance was roughly 23 Å between these two redox centres (Shimada et al., 2017). In our own analysis of horse and *Bos taurus* Cyt. *c* proteins, we found them to be almost identical with 96.2% globally and a local alignment score of 97.1. In 5IY5, since Cyt. *c* and CcO subunit-II are from different sources, one might think that the distances between key residues which are touted to interact are further away than anticipated. We found that the distance between the indole ring of the key CcO residue W104 which was reported to be just 4 Å away from the heme edge (Roberts and Pique, 1999). However, to our surprise, we found that the edge of the indole ring of W104 the almost identical horse Cyt. *c* (to *B. taurus* Cyt. *c*) was at least 9.79 Å way from the heme edge, which is quite a distance. When both horse and bull Cyt. *c* residues were compared, the critical residues which are on the surface of both the proteins are quite similar. Judging from the high degree of conservation, we can expect the interactions of these two Cyt. *c* proteins to CcO from either species to be almost similar, if not identical. Roberts and Pique mutated critical residues mutation and also simulated the interactions. They reported that W104 was critical for ET from Cyt. *c* to CcO. We find that the heme centre is an astounding 14.5 Å away (while the edges of heme and W104 were ~9.8 Å apart; see Figure S2). When very high concentrations of proteins are taken together, it is possible for two proteins to co-crystallize. The path of ET between Cyt. *c* and CcO through was proposed to be >40 Å (41.9) by Shimada et al. (Shimada et al., 2017). It is impractical for ET to occur through such long distances. Till date, very few crystal structures of Cyt. *c*-CcO and Cyt. *c*-CcR complexes exist and hence, we must consider that data were obtained using several methods (including UV-visible spectra) by comparing WT and mutants and by performing chemical modifications.

C. Interactions of Cyt. c with other proteins:

The crystal structure of a 1:1 complex between yeast CcP and Cyt. *c* was determined and the interactions between these two proteins were deemed to be highly specific (Pelletier and Kraut, 1992). When we analyzed the crystal structure of protein-protein complex between Cyt. *c*-CcP (2PCB), residues K5, K7, K8, V11, Q12, K13, Q16, K72, A83, I85, K86, K87, T89 and E90 of Cyt. *c* were found to interact with CcP (See Figure S3 and Table S2). A cation- π interaction between Y-39 in CcP and either R/K at position 13 in Cyt. *c* is recognized as a general binding element in the protein-protein interface; this interaction is deemed to be supported by weak forces between positively charged residues located around the heme of Cyt. *c* and negatively charged surface of CcP (Hunte et al., 2002). In interaction with human neuroglobin (hNgb)- a factor involved in apoptosis prevention, residues Q16, K27, T28, K72, K79, I81, F82 and A83 were found to be involved in the contact interface between Cyt. *c* and hNgb (Tiwari et al., 2018).

Since some of the same residues (which were shown to be necessary for interaction with Cyt. *c*₁ of CcR) are involved in Cyt. *c* binding to CcP, the valid questions to be asked at this juncture are: a) Do Cyt. *c* proteins possess different binding sites for different proteins? b) Does Cyt. *c* compete for binding to different proteins? Just supposing– CcP, CcR and CcP are present at the same time, how does Cyt. *c* know which protein to bind to? We also need to take into account significant diversities of surface proteins' residues (Table 1 and Table S2). The numbers of K and R residues located on the surface and their locations thereof also may be variable in Cyt. *c* of different organisms. Moreover, these 'critical' surface residues' positions are conserved only in mammalian Cyt. *c* proteins and not seen in either plant Cyt. *c* or in the more primitive (bacterial and yeast) counterparts. The relative positions of the coordination sphere also change based on the species, as seen in Table S1. When the Brownian dynamics of association between Cyt. *c* and CcP was analyzed, the two proteins were deemed to achieve plausible ET orientations via the attainment of multiple electrostatically stable encounter complexes, instead of just through a single dominant complex (Northrup et al., 1988). These facts only point to the low probability of ordered, specific and high affinity protein-protein complex formation. Murburn hypothesis does not entail ordered and high affinity protein-protein interactions as prerequisites for ET to occur.

In toto, the exposure of heme to solvent in all Cyt. *c* does not augur well for specific binding-based interactions. If specific interactions were in fact and evolutionarily desired trait, then the heme would have been tucked away and surface complementation would have been evident, along with a preferred ET route for Marcus outer sphere relay.

3. Quantitative aspects of Cyt. *c*'s interaction with its redox partners

From published literature, we present some data showing that mutation of certain surface residues caused changes in thermodynamic and kinetic constants for these proteins' interactions with their redox partners. We compared the values for WT Cyt. *c* vis-à-vis the mutants, as presented in Table 2.

In Cyt. *c* – CcO interactions, WT Cyt. *c* had a K_M of 1.2 μ M and specific lysine mutants such as K13L, K66/87L, K7/8L, K72L and K79L showed 4-10 times increase in K_M values and by this observation, the workers had inferred that these residues were involved in the interaction interface between the proteins (Sato et al., 2016). The acidic residue mutations showed comparatively lesser affects (Witt et al., 1995). Several residues (W143, D188, E189, D151, D195, D214) were altered to obtain single or double mutants of horse CcO, for interaction studies with Cyt. *c* (Zhen et al., 1999). In their work, the authors found no structural changes in the CcO mutants devoid of COOH groups. While the double mutants D188Q/E189N did not alter the K_d and other kinetic constants, single mutants of E148Q, E157Q, D195N, and D214N were found to be less active and had only slightly higher K_M values. This is not expected if the interaction was via a “Cyt. *c* (positive) – CcO (negative) electrostatic” format. Inexplicably, mutants W143A and W143F were found to have severely diminished activity without any significant change in the K_M values for Cyt. *c*. In our analysis, we explored the position of W143 and W145 which were mutated to alanine (Zhen et al., 1999) and found that only *Rhodobacter sphaeroides* CcO subunit-II contained these ‘critical’ residues. They mention that horse CcO subunit-II also contained W143 and W145 but we could not trace these residues in the protein sequence. In fact, positions 143 and 145 in horse CcO subunit-II are V and P residues, respectively. Therefore, we cannot agree with their conclusion that Cyt. *c* was able to bind to an area in the subunit II of CcO which contains W143 and D214, wherein “electrostatic guidance” was provided by residues. A very important finding is that Cyt. *c*'s K_d for WT Cyt. *aa3* was 9.2 mM (the inverse of K_a reported). Such high K_d values would necessitate unrealistic physiological concentrations of Cyt. *c* for binding-based interactions. Interactions between CcR and Cyt. *c* were assessed by a group using the mutants of Cyt. *c*₁, as shown in Table 2 (Tian et al., 2000). In a majority of the mutants, k_{cat} was unaltered but K_M and ET rates were found to be affected. Three different types of effects were observed – (x) increase in K_M and decrease in k_{ET} , (y) decrease in both K_M and k_{ET} and (z) increase in both K_M and k_{ET} . To us, none of these changes in the WT versus mutant

features appear to be highly significant, with respect to mechanistic considerations. More significant is the fact that K_d values are several orders ($\sim 10^4$ times) higher than the K_M values! The classical Michaelis-Menten equation for K_M [$= K_d + (k_2/k_1)$] does not permit this stature and this clearly implies that the conventional binding-based concepts do not apply. We had reasoned such scenarios in P450 and CcO interaction with oxygen, pointing out that this outcome was owing to murburn ET (Manoj, 2018a; Manoj et al., 2019a; Manoj et al., 2016b; Manoj et al., 2019b). This inference is corroborated by the fact that alteration of positively charged amino acid residues could impact its interaction dynamics with a negatively charged DROS like superoxide. We have shown such rationale as the causation for explaining the effects of N-term transmembrane segments containing positively charged amino acid residues (Gideon et al., 2012).

II. Analysis of Cytochrome b_5

1. Overall conservation of Cyt. b_5 structure and its heme coordination environment

When comparing the Cyt. b_5 proteins (see Table 3), the identity scores were above 50-60% in mammalian sources when compared to the Cyt. b_5 counterparts of lower life forms. The heme coordination sphere of Cyt. b_5 contains a hexa-coordinated protoporphyrin-IX (heme) moiety with iron as the central metal atom and two conserved His residues as the axial ligands of heme. Our analyses of a variety of Cyt. b_5 proteins from diverse organisms show even lesser conservation than Cyt. c (Table 3 and Table S3). The redox potential of Cyt. b_5 was measured to be around 20 mv (Vergeres and Waskell, 1995). The protein has an $\alpha+\beta$ architecture; usually, 5 helices and 5 strands are present. The structure of Cyt. b_5 and its heme coordination sphere is presented in Figure 4. Cyt. b_5 has a bulky hydrophilic globular domain which contains heme at one end which is exposed to the solvent. The protein is tethered to the membrane via a C-terminal domain; however, the protein also has a short N-terminal domain. Structurally, Cyt. b_5 has a membrane TMS, a linker domain and the catalytic/heme containing domain (Sobrado et al., 2008). The heme of Cyt. b_5 is well exposed to the surrounding reaction milieu (a protic and oxidative medium that is normally encountered in the microsomal lumen). Our analysis of the Fe-N bonds between the heme iron and nitrogen atoms of the two axial H residues shows that the bond lengths vary between 1.86 to 2.40 Å (Table S3). The varying bond lengths also may be one among several factors which modulate the redox potentials of the protein.

2. Surface residues involved in Cyt. b_5 -redox partner complex formation:

Several theories of Cyt.*b*₅ action have been postulated and there is no consensus on the exact modality of the working of this protein (Schenkman and Jansson, 2003). However, protein-protein binding is considered to be the predominant avenue for intermolecular ET to occur between its partners- CYP and CPR.

A. Specific interactions between Cyt. b₅ and P450s

Electrostatic interactions in the P450- Cyt. *b*₅ interface have been proposed (Qian et al., 2001). Almost always, the P450s contain positively charged surface residues (R and K) which interact with negatively charged (D and E) residues in Cyt. *b*₅. The specific residues in Cyt. *b*₅ that were highlighted for their roles in protein-protein contacts with diverse CYP450s are: E42, E43, E49, V50, N62, D65, V66 and D71. Since CYP450s are structurally diverse, each CYP is believed to have a different set of residues for binding to Cyt. *b*₅. Indeed, a report states that diverse P450s have different Cyt. *b*₅ binding sites (Bart and Scott, 2017). This sort of diversity also can be species-specific and therefore, P450 from a source organism may interact differently with Cyt. *b*₅ or CPR or Cyt. *b*₅R from another source. Since there is low probability for conservation of the CYP-Cyt. *b*₅ interaction site, we question the need for protein-protein contacts in mediating ET.

We examined the residues for their presence on the diverse organisms' Cyt. *b*₅ surfaces and also checked the relative positions of the aminoacid residues to assess if there is some level of residue conservation. When we examined Cyt. *b*₅ proteins from evolutionarily divergent organisms, we found that they did not have D and E residues at the region surrounding the exposed heme iron. The D and E residues were distributed all over the protein (Figure 5). This only means that charge-based interaction may not bring participating proteins in desired orientations, which could reduce the ET path and also tunnel electrons properly.

Using molecular dynamics, a group predicted the interactions between human CYP1A2 and Cyt. *b*₅ complex (Jerabek et al., 2016); the authors theorized that transient complexes formed in the interface of the two proteins would dictate the catalytic outcomes. They found an X-shaped interaction between the antiparallel TMS' of the two proteins was formed within the phospholipid membrane. They identified that several inter-protein salt bridges exemplified the most plausible mode of interaction between the two proteins. The simulations revealed that "Cyt. *b*₅ -sensitive CYPs" such as CYP1A2, CYP2B4, CYP2E1, CYP3A4 and

CYP17A1 had greater propensity for Cyt. *b*₅ binding. In CYP1A2, the key surface residues were found to be R95, R138, R362, K442 and K447. In CYP2B4, the interacting residues were R85, D90, R126, R343, R422 and G245. In CYP2E1, the key contact residues were K87, D92, R127, R344 and Y246. In CYP3A4, aminoacids K91, K96, S131, V350 and K424 were found to be important and finally, in CYP17A1, K88, K83, R126, R347 and S427 were found to be involved in Cyt. *b*₅ binding (Jerabek et al., 2016). In all these cases, there were ionic hydrogen bonds as well as salt bridges between the participating residues. Since such bonding must require a degree of residue conservation and because several reports suggest that weak, charge-based interactions alone are involved in complex formation, we analyzed if different Cyt. *b*₅ partners have common binding sites (wherein –vely charged residues of Cyt. *b*₅ interact with +vely charged residues of Cyt. *b*₅'s redox partners). Both P450s and Cyt. *b*₅R are supposed to interact with Cyt. *b*₅ through some common surface residues (Figures 5,6).

Oligomers/multimers of P450s to form homomeric and heteromeric oligomers has been consistently reported (Davydov et al., 2015; Reed and Backes, 2012; Davydov, 2011), and this can be expected of hydrophobic proteins, as they tend to clump together. The physiological relevance of these P450 dimers and oligomers is not clear per the binding-based perspective. The clustering of these P450s among themselves can theoretically affect the interactions of P450s with the other redox partners such as Cyt. *b*₅ and CPR. In reconstituted systems, various CYP homo- and hetero- dimers such as CYP3A4-CYP3A4, 2C9-3A4, 2C9-2C19, 3A4-2E1, 1A2-2B4, 1A2-2E1, 3A4-1A1 and 3A4-1A2 have been reported (Bostick et al., 2016). Such seemingly endless reports exist in literature and these purported interactions would be counter-productive in explaining the chemistry of P450s in drug metabolism, per the classical perspective. If these structures really do exist in the microsomes and have physiological relevance, how then do P450s recognize their redox partners (CPR/ Cyt. *b*₅)? Also, the relative mobility of P450 becomes constrained by an estimated 50% or higher because of P450-P450 dimers/oligomers (Reed and Backes, 2012, 2017); would this not cause weaker binding of P450 to its redox partners? What signals the switch from P450-P450 interactions to P450-redox partner (CPR and Cyt. *b*₅) interactions? The linker domain of Cyt. *b*₅, a 16 aminoacid peptide, was reported to stimulate CYP2B4 catalysis (Clarke et al., 2004). In some cases, Cyt. *b*₅ inhibits the reaction and contrastingly, in some cases, it enhances the reaction rate – depending on the concentration of Cyt. *b*₅ (Im and Waskell, 2011). Why is this ternary complex of CYP-CPR- Cyt. *b*₅ necessary for efficient

drug metabolism? It could only be expected to pose limitations to kinetic efficiency, by virtue of posing a fastidious mandate.

B. Specific interactions between Cyt. b_5 and CPR/Cyt. b_5R

There are not many reports on Cyt. b_5 -CPR interactions. However, the interactions between these two proteins (CPR and either holo/apo Cyt. b_5) were discovered to be “relatively weak” (Shimada et al., 2005). Reports show that CYP-CPR bind to each other and that Cyt. b_5 either in the holo (heme containing) or apo (devoid of heme) forms was still able to modulate P450 reactions rates. Cyt. b_5 was found to receive electrons from CPR (Guengerich, 2005) using UV-visible spectroscopic investigations and any specific residues of CPR involved in the alleged binding of Cyt. b_5 were not given. Also, one group showed that Cyt. b_5 and CPR competed for a binding site on CYP3A4 (Zhang et al., 2007). Some even hold that the second electron in the P450 cycle comes from Cyt. b_5 , and not CPR. Interestingly, while many deem that CPR is absolutely essential for pumping electrons to CYPs, one report revealed that Cyt. b_5 and Cyt. b_5R in tandem can serve the essential functions of CPR and thereby, act as alternatives of CPR functioning in the reactions of CYP5150A2 (Clarke et al., 2004). NADH Cyt. b_5 reductase (Cyt. b_5R), another flavoprotein, is involved in the reduction of Cyt. b_5 (Dailey and Strittmatter, 1979) in microsomes. The microsomal form of Cyt. b_5R is found in all cell types except in erythrocytes, which contain a soluble form of the enzyme which reduces methemoglobin. Residues in Cyt. b_5 such as E44, E48, E56 and E60 were found to associate with positively charged b_5R residues such as K41, K125, K162 and K163 (Dailey and Strittmatter, 1979; Samhan-Arias et al., 2018).

C. Interactions of Cyt. b_5 with other proteins

Cyt. b_5 reportedly interacts with other proteins such as methemoglobin, myoglobin, Cyt. c ., etc. through electrostatic contacts/charge-based contacts which are mediated by important surface residues (Mauk and Mauk, 1982). If these residues also bind to the same loci of Cyt. b_5 where the other major microsomal redox partners bind to, then there must necessarily be too many competitions which would require transient binding attempts of already confined membrane proteins which are also tethered in clusters as homo and hetero-oligomers. These common surface residues are not specific, because the interactions are mostly charge-based electrostatic forces. If indeed these protein-protein associations occur within the two dimensional microsomal membrane, many competitions and binding promiscuities are

envisaged and these can affect the drug oxidation rates. Multiple sequence alignment of 11 different Cyt. *b*₅ proteins was performed and the alignment is shown in Figure 6. From the data, we can see that most purported residues are poorly conserved (as evidenced by the total absence of any light violet shade which indicates residue conservation).

3. Quantitative aspects of interactions of WT vs. mutant Cyt. *b*₅ proteins

From Table 4, we find that K_d increased from 0.022 (value for WT *b*₅) to 0.332 with the D5A mutant (an increase of ~15.1 times) and 0.152 (~ 6.9 times higher) with the V66A mutant. However, the other mutants did not affect CYP2B4-Cyt. *b*₅ complex formation (Ahuja et al., 2013); in that work, they checked for mutations of critical residues of both 2B4 and Cyt.*b*₅. 2B4 mutants R133A and K139A were found to yield the highest K_d values w.r.t WT (increase of 75 and 27 times, respectively). In another work, the association of Cyt. *c* with Cyt. *b*₅ was explored and it was found that mutating lysine residues increased the K_d values (Qian et al., 2001), quite akin to Cyt. *c*. To us, the demonstration of sub-micromolar values for binding constants of proteins like CYPs and Cyt. *b*₅ is a functional aspect that demonstrates the involvement of reactive diffusible species. Further, we had demonstrated that CYP2E1's kinetics and its substrate-concentration dependence clearly shows the prevalence of one-electron reaction equilibria within milieu (Manoj et al. 2010).

III. Murburn perspective of the functioning of Cyt. *c* and Cyt. *b*₅

Recent reports show that tissue-specific phosphorylation of Cyt. *c* regulates its ET capabilities and thereby, modulates respiration and apoptosis. The specific residues which were found to be phosphorylated are T28, S47, Y48, T58 and Y97; some of these phosphorylations were capable of inhibiting apoptosis (Kalpage et al., 2020). None of these residues have been reported in protein-protein interactions. Since these aminoacids were not deemed to be involved in Cyt. *c*'s interaction with CcR, CcO or Ccp, we are not sure how these phosphorylations can affect the specific interactions between Cyt. *c* and CcR/CcP. Since all these amino acid residues have an –OH functional group, they are susceptible to the murburn phosphoryl transfer. The high amount of lysine in mitochondrial Cyt. *c* could be an evolutionary strategy to capture electrons in superoxide that would otherwise escape the mitochondrion. These observations provide strong support for the murburn theory. The existence of multimeric P450s can be explained to enhance DROS dynamics, as it would deter CYP-CPR complexing. We have shown conclusively that the N-terminal

transmembrane segment (TMS) of cytochrome P450 reductase (CPR) plays a major role in catalysis by anchoring CYP and CPR within the microsomal membrane and it is unlikely that CPR's TMS does not either facilitate hetero-recognition or perform catalytic roles within the active site of CYP450 (Gideon et al., 2012; Manoj et al., 2016a). Similar roles of the membrane anchoring segment of Cyt. *b₅* were proposed to elucidate its role in CYP450 catalysis (Mulrooney et al., 2004; Sergeev et al., 2014; Wu et al., 1994); the two proteins were deemed to interact via weak/non-covalent intermolecular forces.

In Figure 7, the classical binding-based ET and murburn equilibriums based ET are depicted. As seen, the murburn scheme does not need the direct binding of the primary e-donor and final e-acceptor, but this role can be mediated by intermediary DROS and ions. In the light of our long-standing pursuits and current qualitative and quantitative findings, we have demonstrated clearly that proteins like Cyt. *c* and *b₅* are more likely to serve as non-specific electron buffers or generic capacitors. This is an inference which justifies the promiscuity of interaction, evolutionary mandates and Ockham's razor.

CONCLUSIONS

If binding-based long-distance ET via Marcus model was the rationale employed in the protein-protein interactions involving Cyt. *c/b₅*, it would have served the proteins to hide the reactive hemes from bulk solvent and recruit specific electronic circuitry within the protein framework. We could not find any evidence for such a premise. From the comprehensive analysis we had undertaken to explore the involvement of key surface residues in mediating protein-protein interactions between small heme proteins cytochrome *c* and cytochrome *b₅*, we do not find strong evolutionary conservation of the proteins or evidence for complexations. Since the key residues are not necessarily conserved, the extent of binding (even if demonstrated in vitro or in situ) would differ from protein to protein and would be projected to have little physiological relevance. The reaction milieu of Cyt. *c / b₅* are one of the most fundamental redox metabolic routines of life. In evolutionary rationale, strong binding-based interactions and deterministic outcomes are characteristic of two-electron reactions with well-defined substrates. Therefore, we conclude that these proteins work along the murburn logic of generic redox capacitors, which is commonly employed in the redox metabolic routines seen in microsomes, mitochondria and chloroplasts.

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TABLES, FIGURES & LEGENDS

Table 1. Comparison of Cyt. *c* proteins from various sources – Using EMBOSS Water and Needle, the local and global pair-wise alignments of different proteins against *Bos taurus* control (6FF5) were carried out. The entries were chosen to represent the widest possible Cyt. *c* proteins in the evolutionary spectrum.

No	PDB ID	Source of Cyt. <i>c</i>	Global Alignment (%)			Local Alignment (%)		
			Identity	Similarity	Gap	Identity	Similarity	Gap
01	1CCR	<i>Oryza sativa</i> [rice]	57.5	72.6	8.8	63.1	79.6	0.0
02	6K9I	<i>Equus caballus</i> [horse]	96.2	97.1	1.0	97.1	98.1	0.0
03	6C4W	<i>Pichia membranifaciens</i> [yeast, a lower eukaryote]	55.5	67.3	6.4	59.8	72.5	0.0
04	2N9I	<i>Homo sapiens</i> [human]	90.4	94.2	0.0	90.4	94.2	0.0
05	1DW3	<i>Rhodobacter sphaeroides</i> [photosynthetic bacteria]	16.6	27.6	51.0	21.4	38.4	33.9
06	3CYT	<i>Thunnus alalunga</i> [tuna]	82.9	88.6	1.9	84.5	90.3	0.0
07	3CX5	<i>Mus musculus</i> [mouse]	59.6	69.7	5.5	64.4	75.2	0.0
08	2YCC* (starts from -5)	<i>Saccharomyces cerevisiae</i> [yeast]	60.6	70.6	5.5	64.7	75.5	0.0
09	4DY9* (starts from 6)	<i>Leishmania major</i> [parasite]	48.7	61.7	11.3	56.6	71.7	0.0
10	5DFS	<i>Ateles sp</i> [monkey]	87.6	93.3	1.0	88.5	94.2	0.0
11	5C0Z	<i>Rattus norvegicus</i> [rat]	96.2	97.1	1.0	97.1	98.1	0.0

Table 2. Thermodynamic and kinetic studies of WT vs. mutant proteins reported earlier in literature

Interaction	Item	K _d (mM)	K _M (μM)	k _{ET} (10 ⁴ s ⁻¹)	k _{cat} (s ⁻¹)
Cyt. <i>c</i> - CcO [†]	WT Cyt. <i>c</i>	-	1.2 ± 0.1	>1	48 ± 1
	Cyt. <i>c</i> Mutants	-	5-12 ± 1.1	-	62-130 ± 10
Cyt. <i>c</i> - CcO*	WT Cyt. <i>c</i>	-	2.95	-	453
	Cyt. <i>c</i> Mutants	-	4.6 - 5.5	-	250 - 282
Cyt. <i>c</i> – Cyt. <i>aa</i> ₃ [¶]	WT Cyt. <i>aa</i> ₃	9.2	0.63 ± 0.12	-	1700 ± 200
	Cyt. <i>aa</i> ₃ Mutants	4.6 - 22.2	0.38 - 0.98 ± 0.22	-	20 - 1100
Cyt. <i>c</i> - CcR [#]	WT Cyt. <i>c</i>	-	10	6	-
	Cyt. <i>c</i> Mutants <i>x</i>	-	14 - 73	1.2 - 5.0	-
	Cyt. <i>c</i> Mutants <i>y</i>	-	7 - 9	4.4 - 5.7	-
	Cyt. <i>c</i> Mutants <i>z</i>	-	12 - 15	6 - 7	-

[†] (Sato et al., 2016): Mutants - K13L, K86/87L, K72L and K79L

* (Witt et al., 1995): Mutants - D206N, E246Q

[¶] (Zhen et al., 1999): Mutants - E148Q, E157Q, D214/229N, E254A, W143A and W143F

[#] (Tian et al., 2000): Mutants *x* - E74Q, E101K/D102K, E104Q, D109N, E162Q/E163Q, E169Q, E82Q/D83N, D95K, D213K/D214N and E217K; *y* - E97K/E98K, D214K and D220K; *z* - D223N, E56Q and E79Q

*[¶] values entered are derived by averaging the original authors' data reported for biphasic treatment

Table 3. Comparison of Cyt. *b*₅ proteins from various sources (Cytochrome *b*₅4HIN template)

No.	PDB ID	Source of Cyt. <i>b</i> ₅	Global Alignment (%)			Local Alignment (%)		
			Identity	Similarity	gap	Identity	Similarity	gap
01	1CXY	<i>Ectothiorhodospira shaposhnikovii</i> [bacteria]	29.7	51.6	11.0	35.1	59.7	5.2
02	1DO9	<i>Oryctolagus cuniculus</i> (rabbit)	81.9	85.1	12.8	95.1	98.8	0.0
03	1ICC	<i>Rattus norvegicus</i> [rat]	64.4	79.3	5.7	69.1	85.2	0.0
04	1X3X	<i>Ascaris suum</i> [a nematode]	34.5	51.7	11.5	38.0	57.0	6.3
05	2I96	<i>Homo sapiens</i> [human]	72.2	75.0	24.1	95.1	98.8	0.0
06	2IBJ	<i>Musca domestica</i> [house fly]	55.7	70.5	6.8	60.5	76.5	0.0
07	3MUS	<i>Rattus norvegicus</i> [rat]	60.9	78.2	5.7	65.4	84.0	0.0
08	3NER	<i>Homo sapiens</i> [human]	56.5	72.8	10.9	64.2	82.7	0.0
09	3X35	<i>Sus scrofa</i> (wild boar)	85.1	86.2	12.8	97.6	98.8	0.0
10	4B8N	<i>Ostreococcus tauri</i> [green algae]	35.8	51.6	17.9	44.4	63.9	5.6
11	6NZX	<i>Hadesarchaea</i> <i>archaeon</i> [archaeobacteria]	29.4	49.4	11.8	31.2	52.5	8.8
12	6VZ6	<i>Methanococcoides burtonii</i> [archaeobacteria]	29.4	47.1	11.8	31.2	50.0	8.8
13	7BWH	<i>Ramazzottius varieornatus</i> [tardigrade]	24.1	41.1	26.8	31.8	52.9	9.4

Table 4. K_d (μM) data for Cyt. *b*₅ interaction with its redox partners

Interaction→ Phenotype↓	Cyt. <i>b</i> ₅ - CYP2B4 [§]		Cyt. <i>b</i> ₅ - CYP2E1 (PNP) *	Cyt. <i>b</i> ₅ - CYP2E1 (APAP)*	Cyt. <i>b</i> ₅ - Cyt. <i>c</i> †
WT	0.022 ± 0.003		23.1 ± 1.9	1.75 ± 0.1	45.5
Mutant Type 1	0.010-0.017 ± 0.008 (↓)	0.032 ± 0.009 (↑)	12.3 - 18.4 ± 1 (↓)	2.32-3.79 ± 0.2(↑)	19 (↓)
Mutant Type 2	0.032 ± 0.004 (↑)	0.205-0.458 ± 0.07 (↑↑)	-	-	77-91 (↑)
Mutant Type 3	0.152-0.332 ± 0.033 (↑↑)	0.611-1.502 ± 0.18 (↑↑↑)	-	-	192 (↑↑)

[§] (Ahuja et al., 2013) Cyt. *b*₅ mutants – Type I = E42A, E43A, V50A, N62A, D71A; Type II = E49A; Type III = D65A, V66A. CYP2B4 mutants – Type I = H226A; Type II = F135A, R122A, M137A, R126A, K433A; Type III = K139A, R133A

* (Gao et al., 2006) K428A / K434A / K428A+K434A

† (Qian et al., 2001) Type 1 = E48A+E56A+D60A; Type 2 = E44A+E56A+D60A; Type 3 = E48A+E56A+D60A+E44A

Figure 1. Structure of human mitochondrial Cyt. *c* (2N9I): Left - The heme coordinating axial M and H residues are coloured green and cyan, respectively and the two C residues are shown in yellow-. The α -helices are coloured blue and the random coils are coloured white. Right - The coordinating residues and the Fe-N bond lengths are shown.

Figure 2: Surface topographies of Cyt. *c* proteins from diverse organisms – Each of the surface aminoacid residues is coloured by aminoacid; A-light gray, R-red, N-medium blue, D-cornflower blue, C-yellow, Q-orange, E-green, K-dark red, G-black, H-cyan, I-hot pink, L-forest green, P-magenta, F-sandy brown, S-dark khaki, T-tan, W-gold, Y-plum pink and V-dark slate gray. The images represent the halves of cyt. *c* containing the heme moiety, while the rest of the protein is hidden. The large cavity on the protein and the exposed heme residues are a common feature in all the 6 evolutionarily diverse proteins.

Figure 3: Conservation of Cyt. *c* and key surface residues mediating its interaction with redox partners CcR, CcO and CcP. The conserved residues are shaded in light violet. The non-conserved residues are unshaded. Red, blue and green boxes indicate surface residues which are reported to be involved in Cyt. *c* binding to CcR, CcO and CcP, respectively.

Figure 4. Structure of human Cyt. *b*₅ (PDB ID: 2I96): Left - The N- and C-terminal segments are presented on the left and right side of the protein, respectively. The coordinating H residues are coloured cyan. Right - The heme coordinating H residues and the bond lengths are shown.

Figure 5: Various Cyt. *b*₅ proteins, their overall surface structures and their heme cavities – The left side in each panel represents a view of the whole protein and the right side shows the side which contains the heme moiety showing the large cavity which lies at one end of the protein. The heme moiety is coloured red, the coordinating H residues are coloured cyan, while D and E residues are coloured blue and green, respectively. Except in 2I96 (human Cyt.*b*₅), there are no D and E residues near the heme end of the protein. The D and E residues are distributed all over the protein and therefore, there can be no precise orientation to facilitate intermolecular ET. This clearly shows that DROS, and not protein-protein may play a key role in mediating ET.

Figure 6: Sequence conservation of Cyt. b_5 –The pink boxes show interacting residues of Cyt. b_5 with 4-5 different CYP450s and the green boxes which are almost overlapping the same sites as those which the CYPs prefer to bind to, are sites preferred by NADH Cyt. b_5 R.

Figure 7: Schematic to show the classical ET mode and Murburn explanation for Cyt. *c* and *b*₅. While only two examples are shown, the ET donating and accepting roles vis a vis the cytochromes can be penta-coordinated heme (CcO), and in other configurations also.

SUPPLEMENTARY MATERIAL

Roles of cytochromes *c* and *b*₅ in mitochondria and microsomes:

Classical and murburn perspectives

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Table S1. S-Fe and N-Fe bond lengths in various Cyt. *c* proteins

No.	Source of Cyt. <i>c</i>	Cysteine position	Methionine position	Histidine position	Bond length (Å)	
					Methionine S to Fe	Histidine N to Fe
01	1CCR	22, 25	88	26	2.351	2.037
02	6K9I	14, 17	80	18	2.318	2.015
03	6C4W	14, 17	80	18	2.383	2.100
04	5TY3	14, 17	80	18	2.285	1.975
05	2N9I	14, 17	80	18	2.292	1.913
06	1DW3	43, 46	-	47	-	1.877
07	3CYT	14, 17	80	18	2.283	1.957
08	3CX5	19, 25	85	23	2.322	1.937
09	2YCC	14, 17	80	18	2.426	2.011
10	4DY9	28	91	29	2.395	2.031
11	5DFS	14, 17	80	18	2.297	2.023
12	5C0Z	14, 17	80	18	2.293	2.022
13	6FF5	14, 17	80	18	2.310	2.071

Figure S1. Phylogenetic tree for Cyt. *c* proteins from diverse species

Table S2. Surface residue analysis of Cyt. *c* from various organisms

SURFACE RESIDUE ANALYSIS OF Cyt. <i>c</i>											
Amino acids	1CCR	6K9I	6FF5	2N9I	1DW3	3CYT	3CX5	2YCC	4DY9	5DFS	5C0Z
A	1,6,13,23,32,59,64,100,109	15,43,51,83,96,101	15,43,51,83,96,101	43,50,51,96,92,101	6,10,14,15,16,18,20,22,27,49,54,64,70,79,103,107,111	4,15,51,83,96,101	5,8,12,48,56,86,106	-1,3,7,43,51,81,101	7,26,31,62,64,94,103,107	43,51,92,96,101	15,43,44,51,83,92,96,101
	9	6	6	6	17	6	7	7	8	5	8
R	46,99	38,91	38,91	38,91	24,26,53,58,74,80,87,95	38,91	18,43,96	13,38,91	6,16,24,49	8,38,91	38,91
	2	2	2	2	8	2	3	3	4	3	2
N	10,39,41,62,70,78	31,54,70,103	31,54,70,103	31,54,70,103	88,90	22,31,60,61,70	36,57,61,67,68,75,97	31,56,62,63,70,92	37,42,48,81	31,54,70,103	31,54,70,103
	6	4	4	4	2	5	7	6	4	4	4
D	29,74,101	2,50,93	2,50,93	2,62,3	2,21,39,50,73,74,104	2,50,62,93	55,65,95,98	50,60,90,93	13,76,104	2,62,93	2,50,62,93
	3	3	3	3	7	4	4	4	3	3	4
C	Nil	14,17	17	17	89,97	17	19,22,17	Nil	28	14,17	14,1
	0	2	1	1	2	1	3	0	1	2	1
Q	24,36,47,97	12,16,42	12,16,42	16,42	7,33,56,112	12,16,42,92	21,47	16,42	27,100	16,42	12,16,42
	4	3	3	2	4	4	2	2	2	2	3
E	5,15,68,69,98,108	4,21,61,62,66,69,90,92,104	4,21,61,62,66,69,90,92,104	4,21,61,66,69,89,90,104	13,23,62,82,101	21,44,66,69,90	22,26,49,66,71,93,108	-4,21,44,61,66,88,108	15,18,55,65,73,80,101,110	4,21,50,61,66,69,89,90,104	4,21,61,66,69,90,104
	6	9	9	8	5	5	7	7	8	9	7
G	9,31,33,42,45,49,53,85,92	1,24,34,37,41,45,56,77,84	1,23,24,34,37,41,45,56,77,84,89	1,23,24,34,43,74,1,45,56,60,77,84	1,11,35,36,48,55,60,86,94,100	1,24,34,37,41,45,56,77,84	6,28,39,42,46,50,82,88,89	1,23,24,34,37,41,45,77,83,84	12,23,34,35,38,45,52,56,67,88,95	1,23,24,34,37,41,45,56,60,77,84	1,23,24,34,37,41,45,56,60,77,84,89
	9	9	11	11	10	9	9	10	11	11	12
H	26,34	18,26,33	18,26,33	26,33	47	18,26	31,38,44	26,33,39	29	18,26,33	18,26,33
	2	3	3	2	1	2	3	3	1	3	3
I	17,66,83,103	9,57,75,81,85,95	9,57,75,81,85,95	9,11,57,58,81,85,95	9,63,3	57,75,81,85	58,80	53,95	46,69,96,106	9,11,57,58,75,85,95	9,57,75,81,85,95
	4	6	6	7	3	4	2	2	4	7	6
L	43,77	Nil	Nil	94	8,28,30	Nil	14,20,63,90	9,15,58	9,20,43,109,112	Nil	Nil
	2	0	0	1	3	0	4	3	5	0	0
K	12,16,19,21,30,35,61,81,87,95,107	5,7,8,13,22,25,27,39,53,55,60,72,73,86,87,88,99,100	5,7,8,13,22,25,27,39,53,55,72,73,79,86,87,88,99,100	5,7,8,13,22,25,27,53,55,72,73,86,87,88,99,100	37,61,83,102,2	5,7,8,13,25,27,39,53,55,86,87,88,99	4,9,10,16,27,32,59,60,78,84,91,92,94,104,105	-2,4,5,11,22,27,54,55,73,79,86,87,89,99,100	19,22,33,53,61,83,84,90,97,98,113	5,7,13,39,53,55,72,73,86,87,88,99,100	5,7,13,22,25,27,39,53,55,72,73,79,86,87,88,99,100

											99,100
	11	18	18	16	4	13	15	15	11	14	17
M	63	65	65,80	12,65	Absent	80,65	Nil	Nil	86,91	12,65,80	65,80
	1	1	2	2	0	2	0	0	2	3	2
F	3,18,44,90	10,36,46,82	10,36,46,82	10,36,82	29	10,36,82	3,15,41,87	-3,10,36,82	21,44,57,85,93	10,36,46,82	10,36,46,82
	4	4	4	3	1	3	4	4	5	4	4
P	7,8,11,52,84,91,96	44,71,76	44,71,76	30,44,71,76	5,19,38,41,65,68,72,99	71,76	30,76,81	25,71,76	8,10,11,50,72,87,99	71,76	71,76
	7	3	3	4	8	2	3	3	7	2	2
S	42,55,57,104,111	Absent	47	15,47	4,31,42,69,78,91	47,54,100,103	7,45,52,70	2,40,47,65	36,51,60,66,92	15,44	47
	5	0	1	2	6	4	4	4	5	2	1
T	20,27,50,51,58,71,86	19,28,40,47,49,58,63,78,89	19,28,40,49,58,63	19,28,40,49,63,78,102	3,32,34,40,44,45,52,57,59,71,76,98	9,19,40,49,63,78,102	1,13,17,24,54,74,83,101	-5,8,12,19,49,69,96,102	30,32,58,71,89,111	19,28,40,47,49,63,78,102	19,28,40,49,58,63,78,102
	7	9	6	7	12	7	8	8	6	8	8
W	67	Nil	59	59	108,84	33	64	Nil	Nil	59	59
	1	0	1	1	2	1	1	0	0	1	1
Y	56,54,75,82,105	48,64,74,97	48,64,74,97	46,48,67,74,97	12	46,48,67,74,97	51,53,72,79,102	46,48,67,74,97	59,108	48,67,74,97	48,67,74,97
	5	4	4	5	1	5	5	5	2	4	4
V	28,65,89	3,11,20	3,11,20	3,20,83	51,92	3,11,20,28,58,95	25,33,62	20,28,57	14,39,47,54,68,74,77	3,20,83	3,11,20
	3		3	3	2	6	3	3	7	3	3

Figure S2: Crystal structure of horse Cyt. *c* bound to subunit-II of CcO (5IY5)- The two proteins are not very close to each other in this crystal structure.

Figure S3: Complex between Cyt. *c* and CcP (2PCB) – pink is CcP and blue is Cyt. *c*. Interacting residues of Cyt. *c* in close contact with CcP: K5, K7, K8, V11, Q12, K13, Q16, K72, A83, I85, K86, K87, T89, E90. Residues of CcP in close contact with Cyt. *c*: R31, D34, E35, N38, Y39, I40, Q120, A193, A194, N195, N196, V197.

Table S3. S-Fe and N-Fe bond lengths in various Cyt. *b*₅ proteins

No.	Source of Cyt. <i>b</i> ₅	Histidine (H)		Bond length (Å)	
		Position (P1)	Position (P2)	H p1 to Fe	H p2 to Fe
01	1CXY	42	70	2.038	2.095
02	1DO9	39	63	2.010	2.014
03	1ICC	39	63	2.123	2.159
04	1X3X	38	62	2.112	2.128
05	2I96	64	88	2.013	2.403
06	2IBJ	39	62	1.935	2.134
07	3MUS	39	63	2.137	2.150
08	3NER	39	63	1.913	2.187
09	3X35	44	68	1.981	1.971
10	4B8N	48	71	1.860	2.163
11	6NZX	42	61	1.913	1.936
12	6VZ6	42	61	2.090	2.081
13	7BWH	56	80	2.155	2.154
14	4HIN	39	63	2.245	2.159

Figure S4. Phylogenetic tree for Cyt. b_5 proteins from diverse species

Table S4: Analysis of all surface residues present in the selected Cyt. b_5 proteins from diverse organisms.

Amino acids	1CXY	1DO9	1ICC	1X3X	2I96	2IBJ	3MUS	3NER	3X35	4B8N	7BWH
A	16,46,60, 74,75	50	3,13,50,52,54, 67	12,60,65, 66	22,28	10,13,34,50,54, 67,81	3,13,18, 50,52,54	13,50, 67	8,55,59,72, 93	22,54,5 9	27,67,74,77,76, 96,98
	5	1	6	4	2	7	6	3	5	3	7
R	76,80	47,68,84	8,15,28,34,47, 68	Absent	72,93,1 09	9,68,84	8,15,28, 34,68	8,15,28, 34,68	52,73, 89	14	35,73,8 7,92
	2	3	6	0	3	3	5	5	3	1	4
N	41	16,57	16,82	15,18,46	41,82,1 13	15,16,2 7, 28,37,8 6	16,82	16,37	21,22, 62	25,74	28,51
	1	2	2	3	3	6	2	2	3	2	2
D	23,34	1,3,31,53, 60,66,82, 83	1,31,53, 60,66, 78	3,19,25, 51,67	26,56,7 8, 85,91,1 07,108, 124	3,19,31, 53,60,6 6	31,53,60, 66,78,83	31,53, 60,66, 78,83	36,58, 65,71, 87,88	15,29,3 6,40,47, 62,68	36,48,9 9
	2	8	6	5	8	6	6	6	6	7	3
C	53	Absent	Absent	1,77	Absent	Absent	Absent	Absent	Absent	Nil	71
	1	0	0	2	0	0	0	0	0	0	1
Q	55,14,7 9	49	49,72	17,50,75	24,38,7 4	49,73	49,73	-2,49,73	18,54	Absent	14,23,5 3,90
	3	1	2	3	3	2	2	3	2	0	4
E	13,17,5 6, 59,62,6 8	10,11,37, 38,43,44, 48,56,59, 69,78,92	10,11,1 9, 20,38,4 3, 44,48,5 6,	9,10,27, 37	23,27,3 6, 35,62,6 3, 68,69,7 3,	2,11,38, 43,44,4 8,56,59, 69,78, 82,83	10,11,19, 20,38,43, 44,48,56, 59,69	-1,1,10, 11,20, 38,43, 44,48, 56,59, 69	15,16, 42,43, 48,49, 53,61, 64,83	20,27,2 8,65,66, 83	13,20,7 0,97

			59,69,		81,84,9 4, 103,11 7						
	6	12	11	4	14	12	11	12	10	6	4
G	30,44,4 7, 84	41,42,51, 52,62	27,41,4 2, 51,62,7 7	2,26,40, 49	66,67,7 6, 77,87,1 02	41,42,5 1,62,77	27,41,42, 51,62, 77	-3,27, 41,42,51, 62,77	46,47, 56,57, 67,82	50,51,6 0,87	41,58, 59,68,7 9,94
	4	5	6	4	6	5	6	7	6	4	6
H	18,19,2 9, 42,70	15,17,27, 39,63	26,39,6 3,80	14,38	40,42,5 1, 52,64,8 8, 105	26,39,5 7,63	26,39,63, 80	26,39,63, 80	20,31,32,4 4,68,85	24,48,7 1	24,56, 60,80
	5	5	4	2	7	4	4	4	6	3	4
I	83	12,24,75, 76	25,76	22	37,49,1 00, 101,12 0, 123	24,25,4 7	25,76	25,76,79	17,80, 81,92	21,37,5 5,56	62,83, 93,95
	1	4	2	1	6	3	2	3	4	4	4
L	31,64	9,32,70, 79	9,32,36 , 46,71,8 4	45,59,73	34,61,1 04, 112,11 9	36,79	9,36,47, 71,84	9,18,36, 47,71,84	14,30, 41,75, 84	18,45,5 7,70,76, 80,86	44,61, 64
	2	4	6	3	5	2	5	6	5	7	3
K	7,12,35, 50,73,7 7,78,86	2,5,13,14 ,19,28,34 , 72,86,89	14,72,8 5, 87	4,5,8,13, 36,42,47, 57,64,71, 72,74,80, 82	39,44,5 3, 59,97,1 11,114	5,14,18 , 20,52,7 2, 75	14,72,85	14,19,72, 85	10,19, 24,33,39,7 7,91,94	23,43,5 2,53,61, 77,78,9 0,91	37,54, 65,82, 86
	8	10	5	14	7	7	3	4	8	9	5
M	49	91	70	70	21,95	70	70	70	Absent	69	88
	1	1	1	1	2	1	1	1	0	1	1
F	10	35,74	35	34	60,83,9 9	7,23,58	35,58	35	63,79	44,58,6 7	16,75
	1	2	1	1	3	3	2	1	2	3	2
P	8,21,37, 40,43,4 5,69	40,81,90	2,40,65 , 81,86	39,61	65,106, 110,11 5,116	40	2,40,65,8 1,86	40,81,86	86,45	13,46,4 9,72,73, 82,88	40,43,5 7
	7	3	5	2	5	1	6	3	2	7	3
S	20,57,6 5, 71,72	18,20,64, 71,85,88	18,37,5 7, 64	33,54	25,43,4 5,89,12 5,126, 127,12 8	1,64,65 , 85	37,57,64	3,17,55, 57,64,65, 82	23,25, 69,76, 90	17	17,19,2 2,26,29, 39,42,8 1,89
	5	6	4	2	8	4	3	7	5	1	9
T	6,11,36, 58,63,8 5	8,21,33,5 5,65,73,9 3	5,17,21 ,33,55	7,16,32, 53,58,76	33,46,5 8,80,90 ,98,118 ,121,12 2	8,17,33 , 55	5,17,33, 55	2,5,33	13,26, 38,60, 70,78	19,26,4 2,64,81	15,25,3 8,50,55, 69
	6	7	5	6	9	4	4	3	6	5	6
W	61	22	22	21	47	22	22	22	27	31	18
	1	1	1	1	1	1	1	1	1	1	1
Y	38,66,8 1	6,7	6,7,30, 74,75	6,24,35	31,32,5 5	6,30,74	6,7,30,75	6,7,30,75	11,12, 35	39,79	66,83,9 1
	3	2	4	3	3	3	4	4	3	2	3
V	9,39,51	4,45,61	4,45,61 ,79	43,55,63, 68,81	29,70,8 6	4,45,61 ,76,80	4,29,45,6 1,79	0,4,24,29 ,45,52,61	9,50,66	38,84,8 5,89	34,78

	3	3	4	5	3	6	6	7	3	4	2
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Table S5: Key residues of CYPs considered responsible for contacts with Cyt. *b*₅

No	Human Cytochrome P450	Around 120 to 130		Instead of Lysine at 122	Instead of Arginine at 125
		Lysine Position	Arginine Position		
01	2HI4	Nil	Nil	S122	F125
02	2NNH	K121	R125	E122	Nil
03	3E6I	K123	R126	W122	I125
04	3EBS	K125	R123	E122	K125
05	3TJS	K127	R128,R130	E122	E125
06	4GQS	K121	R125	E122	Nil
07	4WNU	Nil	R123,R129	A122	G125
08	5XXI	K121	R124, R125	E122	Nil
09	5VEU	K127	R128,R130	E122	E125