Mechanism of electron transfers mediated by cytochromes c and b₅ in mitochondria and endoplasmic reticulum: classical and murburn perspectives

Daniel Andrew Gideon¹,², Vijay Nirusimhan², Jesu Castin E², Karthik Sudarsha²,³ and Kelath Murali Manoj³

SATYAMJAYATU: THE SCIENCE & ETHICS FOUNDATION, PALAKKAD DISTRICT, KERALA STATE, INDIA; DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS, BISHOP HEBER COLLEGE (AUTONOMOUS), TIRUCHIRAPPALLI, TAMIL NADU, INDIA

COMMUNICATED BY RAMASWAMY H. SARMA

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1. Introduction

Cytochrome c (Cyt. c), a small protein (~12 KDa protein; 25 x 25 x 35 Å dimensions) is believed to be involved in mediating electron transfer (ET) from ubiquinol. Cyt. c reductase (CcR, Cyt. bc₁ complex or Complex III) to cytochrome c oxidase (COX/CcO or Complex IV). It is found in prokaryotes and in mitochondria of algae, fungi, plants and animal cells. Cytochrome b₅ (Cyt. b₅) is a small, and somewhat hydrophobic/acidic cylindrical protein (~16–17 KDa; dimensions of 31 x 37 Å). It is a modulator of electron transfer (ET) from ubiquinol (Oshino et al., 1971). Cytochromes c and b₅ contain a heme prosthetic group. 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. Cytochrome b₅ contains two histidine residues as axial ligands (Battistuzzi et al., 2002).

Cytochromes c and b₅ are deemed to be specific binding-based ET agents. These proteins supposedly bind to their redox partners through charge and topography based intermolecular forces which are mediated by specific surface amino acid residues (Hunte et al., 2002; Nisimoto & Lambeth, 1985). In some cases, even ionic or hydrogen bonds have been reported in the protein-protein interactions (Shimada et al., 2017). In eukaryotes, Cytochrome 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), p66hc (Giorgio et al., 2005), CYPs and apoptosis.

CONTACT
Daniel Andrew Gideon danielandrew.bi@bhc.edu.in
SATYAMJAYATU: THE SCIENCE & ETHICS FOUNDATION, KULAPPULLY, SHORANUR-2 (PO), PALAKKAD DISTRICT, KERALA STATE 679122, INDIA; KELATH MURALI MANOJ murman@satyamjayatu.com SATYAMJAYATU, THE SCIENCE AND ETHICS FOUNDATION, SHORANUR, KERALA, INDIA

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activating factor-APAF-1 (Hüttemann et al., 2011). The other well-known redox partners are cytochrome c peroxidase (CcP), Cyt. b5, Cyt. c reductase/Cyt.bc1 complex, cytochrome c oxidase (McCord & Fridovich, 1969), flavin-dependent sulfhydryl oxidase (Env1), flavocytochrome b2 (Fcb2) and sulfite oxidase (SOX) (Volkov & van Nuland, 2012). The surface residues of CYPs considered essential for Cyt. b5 binding were found to vary from one P450 isozyme to another (Bart & Scott, 2017). Cyt. b5 was reported to bind to myoglobin, α-hemoglobin, several CYPs, stearyl co-enzyme A desaturase, Δ5 & Δ6 desaturase, plasmaplen synthetase, methylsterol monoxygenase, Δ5-Sterol Δ5-desaturase, 17 α-hydroxylase, CYP11A1, C17,20 lyase, metmyoglobin reductase, methemoglobin reductase and also Cyt. c (Qian et al., 1998; Schenkmann & 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 understand their roles at a molecular level 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 b5 held merit.

The purported protein-protein interactions between these cytochromes and their redox partners have been deciphered by researchers using methodologies such as X-ray diffraction, UV-visible, nuclear magnetic resonance (NMR), surface plasmon resonance spectroscopy and kinetics assays (Kandel & Lampe, 2014; Salamon & Tollin, 1996). Also, molecular dynamics simulations are sometimes used to study surface residue contacts between the two proteins when crystal structures of protein-protein complexes are not available. The determination of thermodynamics parameters such as association/dissociation constant (König et al., 1980) and comparison of ET rates between native and mutated proteins were used to ratify the role of key residues in facilitating protein-protein associations between Cyt. c/b5 and their redox partners (Hüttemann et al., 2011). Despite such extensive steps taken toward unraveling the exact mechanism of ET between Cyt. c/b5 and their redox partners, there are significant differences in mechanistic perceptions (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 forces (which do not require exactly the same amino acids) are responsible for the interactions (Mulrooney et al., 2004; Rodgers & Sligar, 1991). Another example is that the proposed stoichiometry of Cyt. cCcP 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 protein-protein interactions between these cytochromes and their redox partners. The protocols for protein contacts map tool (interface plot) to analyze protein-protein contacts (negative control vs. experimental protein-protein complexes), identification of consensus residues involved in contact using COCOMAPS, and mechanical stiffness plot generation are given in the supplementary file, along with the results for these studies.

Justifying our approach: The agenda was to probe for the justification of various criteria meeting the classical scheme, most important of which is: intricate deterministic circuitry within cytochromes that could facilitate electron relay to/from their metal redox centers, via the Marcus outer-sphere ET theory. That is- a conserved circuitry must exist from the metal center Fe of Cyt. c to its periphery and from there, from the periphery of a redox partner (say- Cyt. c1 of Complex III) all the way to its metal center Fe. The logic of such an ‘irreducibly complex’ relay must inherently lie within the framework of the partnering proteins and cannot be left to chance/probability. Therefore, we have analyzed protein sequences without gaps. This is to remove any subjective

2. Methodology

The structures of proteins retrieved from protein data bank were analyzed using popular visualization and various sequence alignment tools (Pettersen et al., 2004; Rice et al., 2000; Waterhouse et al., 2009). UCSF Chimera (Pettersen et al., 2004) and PyMol (DeLano, 2002) were used for surface residue analysis and for preparing images. We employed an array of bioinformatics tools to analyze binding complexes. CONSurf (Glaser et al., 2003) was used to evaluate conservation of key amino acid residues which are reported in literature to aid in protein-protein contact. Protein Contact Maps tool, COCOMAPS (Vangone et al., 2011), and PRODY (DCCM and MechStiff) (Bakan et al., 2011) were used further to generate heat maps of residue contact between the cytochromes and their redox partners. The protocols for protein contacts map tool (interface plot) to analyze protein-protein contacts (negative control vs. experimental protein-protein complexes), identification of consensus residues involved in contact using COCOMAPS, and mechanical stiffness plot generation are given in the supplementary file, along with the results for these studies.
bias and the residues were compared in continuum from N to C terminus. Insights gained from our earlier work on redox partnering in liver microsomal xenobiotic metabolism is crucial in this regard. Aligning short amino acid chain sequences with a few secondary structures using various aligning codes pre-supposes conservations and give subjective outcomes. For seeing a simple example, please refer Item 1, supplementary information (SI), which compares the gap analysis with three alignment programs (ClustalW, T-Coffee and MUSCLE) for Cyt. c. Regardless; we have also conducted alignment with gaps and surface residue analyses from the crystal structures of the cytochromes, to see if secondary conservation mechanisms worked for retaining topology of the same amino acid residue at the respective locus. Further, if the proteins docked with affinity to give and take electrons, then there must be a complementary surface on the respective redox partners, and this too must be conserved.

3. Results and discussion

Affinity binding cannot be a one-point contact phenomenon, as it is a low-probability outcome. Furthermore, there must be a distinguishing of the surfaces for the oxidized and reduced protein with their redox counterparts. That is- only oxidized cytochrome c should bind to Complex III whereas only reduced cytochrome c should bind to Complex IV. We scouted for such mechanistic requisites of complementary facets (required for the classical mechanism) in diverse redox protein complexations. Based on kinetic arguments, we have repeatedly advocated that observation of weak/transition bindings/interactions or co-crystallizations in/from highly concentrated pure solutions (particularly, at low ionic strengths) does not connote that these kinds of interactions are physiologically relevant (Manoj, 2006, 2018; Manoj & Hager, 2008; Manoj et al., 2010a, 2016a, 2016b, 2016c, 2019a). At the outset, we affirm that our approach does not overlook the evidence presented by the classical interpretations (of interactions seen at high protein concentrations) but aims to be comprehensive, and attempts to make inferences inclusive of all reported data.

3.1. Analysis of cytochrome c

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

Cyt. c comprises of a single polypeptide chain containing usually 100–104 amino acid residues in higher organisms. However, in other organisms, it can possess anywhere from 85–135 amino acids (Salemme, 1977). While a majority of Cyt. c proteins possess five α-helices, some of them contain a small percentage of β-sheets. 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, by two H residues. c-type cytochromes possess significant structural diversities – for example, Cyt. c and Cyt. C566* contain other kinds of folds (Barker & Ferguson, 1999). However, heme is tethered via two conserved Cys residues in c-type cytochromes and these proteins have similar ET roles. Alteration of the coordinating S-atom of methionine residues with substituents such as N, O and Se changed the ET/kinetic characteristics, spectroscopic signatures and redox potentials of Cyt. c (Battistuzzi et al., 2002). Hence, the coordination sphere 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 mV (Battistuzzi et al., 2001, 2002). 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 Fe-S bond length alteration was deemed to provide reorganizational energy for fast ET between Cyt. c and its partners (Cheng et al., 1999). Other factors influencing 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, Item 2, SI. The bond lengths between 5-atoms of the M residue and heme-Fe range from ~2.3 to 2.4 Å, while the H residue N-atom to heme-Fe bond lengths are in the range of ~1.9–2.1 Å. From this data, it is apparent that there are significant diversities in heme-Fe bond lengths of both axial ligand atoms. The structure of human Cyt. c and its heme coordination sphere is presented in Figure S1, Item 2, SI. 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. With Bos taurus Cyt. c (6FF5) as 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 S2, Item 2, SI). This shows that the overall amino acid 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, Cyt. c sequences of Rhodobacter, Leishmania and Pichia have much lower scores against the Bos taurus control. These details are available in Table S2, Item 2, SI. The topology (of select proteins) and sequence comparison are shown in Figures 1 and 2 respectively. Evidently, the argument for evolutionary conservation of surface residues and topological recognition is unsupported.

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

3.1.2.1. 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 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 mOxPhos protein 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 mediating protein-protein contacts. Mitochondrial Cyt. c proteins possess a high % of K residues (around 16–19 K out of a total of 104 amino acids). Several independent experiments had confirmed the important roles of residues K8, 13, 27, 72, 86 and 87 in facilitating Cyt. c binding to Cyt. c1 of the Cyt. bc1 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 via electrostatic interactions with the negatively charged surface of CcR. In another report on the crystal structure of a CcR complex with Cyt. c, the heme-heme (center) distance was 17.4 Å and the vinyl groups of the two porphyrins were only 4.5 Å apart (Lange & Hunte, 2002). In that work, nonpolar interactions with A103, F230, M233 and E235 (in Cyt. c1) through T12, R13, V28, A81 as well as ‘potential polar interactions’ prof figured by K79 and K86 (with A164 and E235 of Cyt. c1) were shown to be involved in close contact (<4 Å) between Cyt. c1 and Cyt. c. However, in the same paper, the authors also suggested that the stabilization of the interaction between Cyt. c-c1 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 & Hunte, 2002).

In our analyses of the surface residues of 12 different Cyt. c proteins across several species, among the 10+ K residues which are located on the surface (except in 1DW3, which has just 4 surface K residues and mainly R residues instead), the residues K8, 13, 27, 72, 86 and 87 were found to be conserved in Cyt. c proteins of higher (esp. mammalian) organisms. These residues were entirely absent in 1DW3 & 4DY9, while only K87 was present in 1CCR. Also, all (>10) K residues were not found on the surface of Cyt. c in other species (e.g. 5DFs and 3CX5) (please see Tables S2 and S3, Item 2, SI for more details on all surface residues of Cyt. c present in diverse species). The overall topography of a few Cyt. c proteins (Figure 1) and analyses of their surface residues (as per literature discussed herein) is given in Figure 2 to provide a visual representation of residue conservation. 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 amino acids deemed important (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. Also, V28 is found on the surface of only 1CCR and 3CYT, while A81 is present only on the surface of 1YCC. Apart from the analyses without applying gap penalties, alignment was carried out using ClustalW and the differences in conservation of key residues are shown in Figure S3 and Table S4, Item 2, SI. Again, from that analysis, we can conclude that not all of these residues which are deemed to be required for Cyt. \textsubscript{c} interaction with its redox partners are conserved.

Another report shows that \textit{Arabidopsis thaliana} (plant) mitochondrial Cyt.\textsubscript{c} possesses two different binding sites for Cyt.\textsubscript{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. \textsubscript{c} and hence, were referred to as ‘active’ contact residues (Moreno-Beltr\textsubscript{a}n et al., 2014). However, they discovered that around 47 different residues of Cyt. \textsubscript{c} were ‘passive’ contact residues because of their >50% solvent accessibility. It is quite logical to doubt if almost half of the amino acids of a protein aid in interaction and binding with another protein. Moreover, the same study reported the presence of two distinct binding sites for both oxidized and reduced Cyt. \textsubscript{c} based on their ab-initio Brownian dynamics calculations and NMR-driven docking computations. While the first site is a non-productive distal site, the other is a functional/proximal site wherein the heme groups of the two proteins were reckoned to be close enough to permit direct ET (Moreno-Beltr\textsubscript{a}n et al., 2014). The literature is filled with such examples where several different residues have been proposed as surface contact points involved in Cyt. \textsubscript{c} docking to Cyt. \textsubscript{c}. 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. \textsubscript{c} with its partners (with respect to their proposed functionality) and therefore, some have attempted to identify the specific interactions using molecular dynamics and protein-protein docking studies (Roberts & Pique, 1999). Therefore,
the absence of effective surface residues-based binding rationale is evident for Cyt. c reduction process.

3.1.2.2. Surface residues supposedly involved in Cyt. c-CcO complex formation. CcO/Complex IV is also known to possess surface residues which have high affinity for binding to 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-Q121, A83-E157, A83-D158, K72-L136, P76-L136, G77-L136, K87-L166 and K86-S67 (Roberts & Pique, 1999). Another 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). Yet 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 Cyt. c and subunit II of CcO (which contains 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 with subunit II of CcO, while other minor contacts were observed with subunits VIb and I. Intermolecular interactions between CcO and Cyt. c were found to be: (i) salt bridges between K8 (Cyt. c) and D139 (CcO subunit II) as well as K87 (Cyt. c) -D119 (CcO subunit II), and (ii) hydrogen bonds between Cyt. c-CcO subunit II (Q12-D139 and K13-Y105/ 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 lowered ET rates obtained upon chemical modifications (K8, K13 and K87) and site directed mutagenesis (to produce mutants K13L, K86L and K87L). Till date, very few crystal structures of Cyt. c-CcO and Cyt. c-CcR complexes exist. We analyzed the crystal structure of a horse Cyt. c- Bos taurus CcO complex (PDB ID: 5IYS) and found that the distance between heme iron of Cyt. c and CuA of CcO was 22.3 Å. 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 equine and bovine Cyt. c proteins, we found them to be almost identical with global and local alignment scores of 96.2% and 97.1%, respectively. In 5IYS, Cyt. c and CcO subunit-II are from different sources (horse and Bos taurus). One might argue that the distances between key surface residues of the two proteins may not be ideal because these two proteins are from different sources. The distance between the indole ring of the key CcO residue W104 was reported to be just 4 Å away from the heme edge of Cyt. c (Roberts & Pique, 1999). To our surprise, we found that the edge of the indole ring of W104 and the almost identical horse Cyt. c (to B. taurus Cyt. c) was at least 9.79 Å way from the heme edge. 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. Another study (Roberts & Pique, 1999) involved mutation of critical residues and also simulation of the intermolecular interactions. They reported that W104 was critical for ET from Cyt. c to CcO. We find that the heme centre is 14.5 Å away (while the heme edge of Cyt. c and W104 of CcO were ~9.8 Å apart; see Figure S4, Item 2, SI). Although the path of ET between Cyt. c heme and subunit II of CCO is about 7 Å (3.6 Å to Y105 + 4.0 Å from Y105 to CuA), it is quite unlikely that the electron from Cyt. c traverses a very large distance of ~4 nm through the entire complex IV, until it reaches the last redox component of oxygen (Shimada et al., 2017). We deem that it is univiable (thermodynamically, kinetically and mechanistically) for such serial outer sphere ET to occur viably above long distances. Additionally, we performed CONSURF analysis of several Cyt. c proteins to identify whether the amino acids on the surface of the heme cavity (which are known to be involved in partner binding) are indeed conserved. Relatively low CONSURF scores (5 or below 5) were obtained (See all the materials in Item 2, SI). The above findings can be explained by considering that for deriving crystals to study via X-ray crystallography, when very high concentrations of proteins are taken together, it is possible for two proteins to co-crystallize due to the ‘unnatural’ conditions.

3.2. Interactions of Cyt. c with other proteins

The crystal structure of a 1:1 complex between yeast CcP and Cyt. c was determined (Pelletier & Kraut, 1992) and the interactions between these two proteins were deemed to be highly specific. 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 S4 and Table S3, SI). 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 the negatively charged surface of CcP (Hunte et al., 2002). In its interaction with human neuroglobin (hNgb, a factor involved in apoptosis prevention), Cyt. c surface residues—Q16, K27, T28, K72, K79, I81, F82 and A83 were found to be involved (Tiwari et al., 2018).

Since some of the same residues (which were shown to be necessary for interaction with Cyt. c) 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) If Cyt. c recognizes multiple partners through charge-based interactions, does Cyt. c compete for binding to these different proteins? Just supposing—CcP, CcR and CcO are present at the same time, how does Cyt. c recognize which protein to bind to? We also need to take into account significant diversities of surface residues in the proteins analyzed in this work (Tables S2 and S3, Item 2, SI). The numbers of K and R residues and their location on the surface also may vary in Cyt. c of different organisms. Moreover, these ‘critical’ positions of the surface
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, SI. 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). Analysis of perturbations in CcP-Cyt. c complexes, instead of just through a single dominant complex deemed to achieve plausible ET orientations via the attain-

In toto, the exposure of heme to solvent in all Cyt. c proteins compared in this work does not augur well for specific binding-based interactions. If specific interactions were in fact an 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.

3.2.1. Quantitative aspects of Cyt. c 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 the interaction of these proteins with their redox partners. We compared the values for WT Cyt. c vis-à-vis the mutants, as presented in Table 1. In Cyt. c – CcO interactions, WT Cyt. c had a $K_M$ of 1.2 ± 0.1 μM 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 less dramatic effects (Witt et al., 1995). Several residues (W143, D188, E189, 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 which were 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, 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 disagree with their conclusion that Cyt. c was able to bind to an area in subunit II of CcO which contains W143 and D214, wherein ‘electrostatic guidance’ was provided by those residues. A very important finding is that $K_d$ of Cyt. c for WT Cyt. c αα3 was 9.2 mM (which is the inverse of $K_M$ reported). Such high $K_d$ values would necessitate unrealistic physiological concentrations of Cyt. c to facilitate binding-based interactions. Interactions between CcR and Cyt. c were assessed by a group using the mutants of Cyt. c, as shown in Table 1 (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. These 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 $k_{ET}$. We do not perceive these changes in the WT versus mutant as highly significant, with respect to mechanistic considerations. (While

### Table 1. Thermodynamic and kinetic studies of WT vs. mutant proteins reported earlier in literature.

| Interaction | Phenotype | Item | $K_d$ (μM) | $K_M$ (μM) | $k_{ET}$ ($10^9$ s⁻¹) | $k_{cat}$ (s⁻¹) |
|-------------|-----------|------|------------|------------|-------------------|-----------------|
| Cyt. c - CcO⁰ | WT Cyt. c | – | 1.2 ± 0.1 | >1 | 48 ± 1 |
| Cyt. c Mutants | – | 5–12 ± 1.1 (1) | – | 62–130 ± 10 (1) |
| Cyt. c - CcO⁰ | WT Cyt. c | 2.95 | – | – | 453 |
| Cyt. c Mutants | – | 4.6 – 5.5 (1) | – | 250 – 282 (1) |
| Cyt. c – Cyt. αα3 | WT Cyt. αα3 | 9.2 | 0.63 ± 0.12 | – | 1700 ± 200 |
| Cyt. c – Cyt. αα3 Mutants | 4.6 – 22.2 (1) and (2) | 0.38 – 0.98 ± 0.22 (1) | – | 20 – 1100 (1) |
| Cyt. c - CcR⁰ | WT Cyt. c | – | 10 | 6 | – |
| Cyt. c Mutants x | – | 14 – 73 (1) or (2) | 1.2 – 5.0 (1) | – |
| Cyt. c Mutants y | – | 7 – 9 (1) | 4.4 – 5.7 (1) | – |
| Cyt. c Mutants z | – | 12 – 15 (1) | 6 – 7 (1) | – |

Note. In the mutants, upward or downward arrows indicate a respective increase or decrease in value.

aSato et al., 2016: Mutants - K13L, K66/87L, K7/8L and K79L.

bWitt et al., 1995: Mutants - D206N, E246Q.

cZhen et al., 1999: Mutants - E148Q, E157Q, D214/229N, E254A, W143A and W143F.

dTian 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 are derived by averaging the original authors’ data reported for biphasic treatment.
3.3. Analysis of cytochrome b5

3.3.1. Overall conservation of Cyt. b5 structure and its heme coordination environment

When comparing the Cyt. b5 proteins (see Table S1, Item 3, SI), the identity scores were above 50–60% in mammalian sources when compared to the Cyt. b5 counterparts of earlier life forms. The heme coordination sphere of Cyt. b5 contains a hexa-coordinated protoporphyrin-IX (heme) moiety with iron as the central metal atom and two conserved H residues as the axial ligands of heme. Our analyses of several Cyt. b5 proteins from diverse organisms show much lesser conservation than Cyt. c (Tables S4–S6, Item 3, SI). The redox potential of Cyt. b5 was measured to be ~20 mv (Vergeres & Waskell, 1995). The protein has an α + β architecture; usually, five helices and five strands are present. The structure of Cyt. b5 and its heme coordination sphere is presented in Figure S5, SI. Cyt. b5 has a hydrophilic globular domain which contains a solvent-accessible heme at one end. The protein is anchored to the membrane via a C-terminal domain and the protein also has a short N-terminal domain. Structurally, Cyt. b5 has a membrane TMS, a linker domain and the catalytic/heme containing domain (Sobrado et al., 2008). The heme of Cyt. b5 is well exposed to the surrounding reaction milieu (an oxidative medium that is normally encountered in the endoplasmic reticulum). 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 ~1.9 and 2.4 Å (Table S2, Item 3, SI). The varying bond lengths may also be one among several factors which modulate the redox potentials of the protein. The phylogenetic tree of the various Cyt. b5 proteins explored in this study is shown in Figure S2, Item 3, SI. At the outset, the protein shows significant diversity and little evolutionary conservation that would be necessary for the classical ET scheme.

3.3.2. Surface residues involved in Cyt. b5-redox partner complex formation

Several theories of Cyt.b5 action have been proposed and there is no consensus on the exact modality of the working of this protein (Schenkman & Jansson, 2003). However, protein-protein binding is considered to be the predominant avenue for intermolecular ET to occur between its partners-CYP and CPR.

3.3.2.1. Specific interactions between Cyt. b5 and P450s.

Electrostatic interactions in the P450-Cyt. b5 interface have been proposed (Qian et al., 2001). Almost always (in protein-protein interactions of P450 with its partners), the P450s contain positively charged surface residues (R and K) which interact with negatively charged (D and E) residues in Cyt. b5. The specific residues in Cyt. b5 that were highlighted for their roles in protein-protein contacts with diverse CYP450s are: E42, E43, E49, V50, N62, D65, V66 and D71. Table S6, SI shows all the surface residues of various Cyt. b5 proteins which were compared in this study. Since CYP450s are structurally diverse, each CYP is believed to have a different set of residues for binding to Cyt. b5. Indeed, a report states that diverse P450s have different Cyt. b5 binding sites (Bart & Scott, 2017). This sort of diversity also can be species-specific and therefore, P450 from a source organism may interact differently with Cyt. b5 or CPR or Cyt. b5R from another source. Since there is low probability for conservation of the CYP-Cyt. b5 interaction site, it is only natural that the need for protein-protein contacts in mediating ET is questioned.

We examined the residues for their presence on the surface of Cyt. b5 from diverse organisms and also checked the relative positions of the amino acid residues to assess if there is some level of residue conservation. Our analysis shows that Cyt. b5 proteins from evolutionarily divergent organisms (in all of the b5 proteins analyzed) did not possess any D and E residues at the region surrounding the exposed heme iron. The D and E residues were distributed all over the protein, instead of just on the surface of the heme cavity (Figure 3). This only means that charge-based interaction may not bring participating proteins in desired orientations (which is needed by the classical scheme, to reduce the ET path and also tunnel electrons properly).

Using molecular dynamics, a group predicted the interactions between human CYP1A2 and Cyt. b5 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 that 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. b5-sensitive CYP’s’ such as CYP1A2, CYP2B4, CYP2E1, CYP3A4 and CYP17A1 had greater propensity for Cyt. b5 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, amino acids
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. b₅ binding (Jerabek et al., 2016).

Table S7 shows the surface residues of some P450s which were found to be necessary for redox partner recognition. In all these cases, there were ionic hydrogen bonds as well as salt bridges between the participating residues. Such bonding must require a high degree of residue conservation and several reports suggest that weak, charge-based interactions alone are involved in complex formation. Therefore, we analyzed if different Cyt. b₅ partners have common binding sites (wherein –vely charged residues of Cyt. b₅ interact with positively charged residues of their redox partners). Both P450s and Cyt. b₃R are supposed to interact with Cyt. b₅ through some common surface residues. A visual analysis of the surface topology and amino acid distribution profiles of various Cyt. b₅ proteins does not support the requisites of the classical ET scheme (Figures 3 and 4).

P450-P450 interactions which lead to homomeric and heteromeric oligomer formation have been consistently reported (Davydov et al., 2015; Reed & Backes, 2012), 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. b₅ and CPR. In reconstituted systems, various CYP homo- and heterodimers 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). The multimeric associations of CYPs would be counter-productive in explaining the chemistry of P450s in drug metabolism, per the classical perspective because it would lower the probability of their interactions with their interaction partners like Cyt. b₅ and CPR. If these structures had physiological relevance, how then do P450s recognize their redox partners (CPR/Cyt. b₅)? Also, the relative mobility of P450 becomes constrained by an estimated 50% or higher because of P450-P450 dimers/oligomers (Reed & Backes, 2012, 2017); would this not cause weaker binding of P450s to their redox partners? What signals the switch from P450-P450 interactions to

Figure 3. Topology of Cyt. b₅ proteins taken in the study. The various source organisms of the b₅ proteins and the corresponding PDB IDs are as follows: 4HIN - Bos taurus, 1CXY - Cyt. b₅₈ a prokaryotic homologue of Cyt. b₅ in Ectothiorhodospira shaposhnikovii, 1DO9 - Oryctolagus cuniculus, 2IBJ – Musca domestica, 2I96 - Homo sapiens, 3X35 - Sus scrofa, Hadesarchaeae archaeon YNP_N21, 7BWH - soluble b₅ from Ramazzottius varieornatus and 1X3X – Ascaris suum. Colour codes: A - black, N - purple, D - dark blue, E - light green, Q - navy blue, G - orange, H - cyan, P - magenta, I - dark slate gray, L - forest (dark) green, V - dim gray, S - baby pink, T - medium purple, R/K - red, F - yellow and Y - golden yellow. For counts of the conserved residues represented on this surface, check Table S6, Item 3, SI. Note: In the panels above, the distal side of the Cyt. c proteins are hidden to show the heme and open channel clearly. The images were generated using UCSF Chimera.
P450-redox partner (CPR and Cyt. b) interactions? The linker domain of Cyt. b, a 16 amino acid peptide, was reported to stimulate CYP2B4 catalysis (Clarke et al., 2004). In some cases, Cyt. b inhibits the reaction and contrastingly, in some cases, it enhances the reaction rate depending on the concentration of Cyt. b (Im & Waskell, 2011). Why is this ternary complex of CYP-CPR-Cyt. b necessary for efficient drug metabolism? It could only be expected to pose limitations to kinetic efficiency, by virtue of posing a fastidious mandate. Also, evolutionarily, if protein-protein interactions between two intended partners (say, a and b) were the physiological 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. All these queries can be efficiently addressed by considering the obligatory involvement of DR(O)S like superoxide. A high concentration of superoxide would sequester the one-electron equivalents, thereby proving inhibitory (Manoj et al., 2010a, 2010b, 2016a, 2016b). Involvement of DROS can also explain P450 clumping, as it enables them to better scavenge the one-electron equivalents released (Manoj and Parashar, 2021).

3.3.2.2. Specific interactions between Cyt. b and CPR/Cyt. b.R. There are not many reports on Cyt. b-CPR interactions. However, the interactions between these two proteins (CPR and either holo/apo Cyt. b) were found to be ‘relatively weak’ (Shimada et al., 2005). Reports show that CYP-CPR bind to each other and that Cyt. b either in the holo (heme containing) or apo (devoid of heme) forms was still able to modulate P450 reactions rates. Cyt. b was found to receive electrons from CPR (Guengerich, 2005) using UV-visible spectroscopic investigations, but the residues involved in binding of Cyt. b were not given. Also, one group showed that Cyt. b 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, and not CPR. Interestingly, while many deem that CPR is absolutely essential for pumping electrons to CYPs, one report revealed that Cyt. b and

![Sequence conservation of Cyt. b5.](image-url)
Cyt. b₃R in tandem can serve the essential functions of CPR and thereby, act as alternatives of CPR functioning in the reactions of CYP150A2 (Clarke et al., 2004). NADH Cyt. b₃ reductase (Cyt. b₃R), another flavoprotein, is involved in the reduction of Cyt. b₃ (Dailey & Strittmatter, 1979) in microsomes. The microsomal form of Cyt. b₃R is found in all cell types except in erythrocytes, which contain a soluble form of the enzyme which reduces methemoglobin. Residues in Cyt. b₃ such as E44, E48, E56 and E60 were found to associate with positively charged b₃R residues such as K41, K125, K162 and K163 (Dailey & Strittmatter, 1979; Samhan-Arias et al., 2018).

### 3.3.2.3. Interactions of Cyt. b₃ with other proteins.

Cyt. b₃ 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 & Mauk, 1982). If these residues also bind to the same loci of Cyt. b₃ where the other major microsomal reductase partners bind to, then 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. b₃ proteins was performed and the alignment is shown in Figure 4.

Additionally, we aligned Cyt. b₃ sequences by applying gap penalties and did not find the residues of Cyt. b₃ - which are known to facilitate protein-protein binding, to be highly conserved (see Tables S5–S7 of Item 3, SI and the relevant Figures associated with that data). 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). In our experimental works, we have elaborated on the ET abilities of Cyt. b₃ in various reaction environments to show that the interaction is rather non-specific and easily modulated by additives within the milieu, which supports the murburn view of ET (Manoj et al., 2010a, 2016c).

### 3.3.3. Quantitative aspects of interactions of WT vs. mutant Cyt. b₃ proteins

In Table 2, it can be seen that Kₐ increased from 0.022 (value for WT Cyt. b₃) 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. b₃ complex formation (Ahuja et al., 2013); in that work, the authors checked for mutations of critical residues of both 2B4 and Cyt b₃. 2B4 mutants R133A and K139A were found to yield the highest Kₐ values w.r.t WT (increase of 75 and 27 times, respectively). In another work, the association of Cyt. c with Cyt. b₃ was explored and it was found that mutating lysine residues increased the Kₐ values (Qian et al., 2001), quite akin to Cyt. c. To us, the demonstration of sub-micro-molar values for binding constants of proteins like CYPs and Cyt. b₃ is a functional aspect that demonstrates the involvement of reactive diffusible species. Further, we had demonstrated that the kinetics of CYP2E1 and its substrate-concentration dependence clearly shows the prevalence of one-electron reaction equilibriums within milieu (Manoj et al., 2010a).

### 3.3.4. Murburn perspective for the functioning of Cyt. c and Cyt. b₃

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 amino acids were not considered to be involved in the interaction of Cyt. c 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. The aminoacids lysine, proline, histidine and glutamate are known to be amongst the bevy of aminoacids that are most affected by ROS and oxidative stress (Nimse & Pal, 2015). In an analysis of SOD activity modulations by chalcones (Soulère et al., 2003), Soulère et al.
suggested that activity decrease in SOD caused by chalcones could be attributed to covalent modification of residues like Lys29 or Arg170 located at the entry channel leading to the active metal ion of the SOD active site. They stated that these two residues were responsible for ‘electrostatic control of substrate diffusion’. Lys and Arg are positively charged amino acids and therefore, these residues can also attract radicals and ROS. Researchers have shown that Lys residues in SoxR are critical for sensitivity of SoxR to superoxide (Fujikawa et al., 2017). These observations provide strong support for the murburn theory. The existence of multimeric P450s would deter CYP-CPR complexing but enhance DROS dynamics (Manoj et al., 2020c, 2020d). We have shown conclusively that the N-terminal transmembrane segment (TMS) of CPR plays a major role in catalysis by anchoring CYP and CPR within the microsomal membrane and it is unlikely that the TMS of CPR facilitates either hetero-recognition or performs 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 the light of the explorations reported herein and the literature available, we propose the murburn perspective as a more holistic mechanism, compared to the classical binding-based ET (Figure 5). 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 this regard, we have amply demonstrated the involvement of DROS/ions in ET processes and the ability of the reaction milieu to substitute the roles of these proteins with various other redox active small and large molecules that differ significantly from the original redox protein (Gade et al., 2012; Manoj, 2006; Manoj et al., 2010a, 2010b, 2016a, 2016b, 2016c; Parashar et al., 2014, 2018; Parashar & Manoj, 2012). Examples of how the diffusible species could relay electrons are given within the equations of Figure 5. It can be seen that the diffusible species mediated ET is thermodynamically facile and since they involve bimolecular collisions of small atoms or molecular species (which can directly access the hemes of the cytochromes), it is effected extremely fast, in the range of $10^{10} \text{M}^{-1}\text{s}^{-1}$. (For the details of thermodynamic calculations employed herein, please refer our earlier reports (Parashar & Manoj, 2021; Manoj & Bazhin, 2019; Manoj et al., 2020b, 2020e). These considerations also explain the modulatory effects of positively charged residues on the redox proteins (e.g. high density of lysine on Cyt. c heme side), as they may affect superoxide interaction dynamics. Also, the enhancement of outcomes observed at lower pH is reasoned with these theoretical explanations.

3.4. Support from our earlier studies, particularly on plastocyanin and P450s

Recently, we had shown that the surface topography of plastocyanin (of chloroplasts), the plant protein supposed to carry out homologous function of cytochrome c (in mitochondria), was not conserved (Gideon et al., 2020). The inference from multitude of experimental and theoretical arguments supported the murburn modality of ET within this system. Since some peers did not quite grasp our approach, we have now followed through the works on this protein with multitudes of other methodologies, and the results are given in Item 4, SI. It can be seen that our inferences are fully corroborated with the new supplemental data. Further, while the binding-based or affinity-based ETs necessitate residue conservation, what matters more is if the purported residues are located on specific regions of PC when they make contact with their redox partners. In the main text figures, we had/have used JalView, without applying gap penalties (ClustalW/T-Coffee). This approach stems from our (unreported) prior experience with conservation analysis in some membrane proteins of the xenobiotic metabolism system. We found therein that when there are very few secondary structures in a peptide with $\sim 100$ residues, it makes little purpose to screen or align with gaps, as this exercise overtly assumes conservation. Our investigations showed that contrary to earlier supposed belief systems, there was no real conservation logic for ETs based on protein-protein complexations that were erroneously assumed to be mediated between N-terminal
Table 3. Contact points (ET pathway/route) for purported affinity-based ET.

| System          | Donor 1                | Acceptor 1            | Donor 2                | Acceptor 2            |
|-----------------|------------------------|-----------------------|------------------------|-----------------------|
| Mitochondria    | Red. Comp. III - Y95   | Ox. Cyt. c (K?)       | Red. Cyt. c - K13      | Ox. Comp. IV - W104   |
|                 | - Direct heme c-heme c | (Shimada et al., 2017) | (Scharlau et al., 2019)/K86 | (Scharlau et al., 2019) |
|                 | contact (Hunte et al., 2002) |                       |                        |                       |
| Microsomes      | Red. CPR (??)          | Ox. Cyt. b5 (??)      | Red. Cyt. b5 Heme propionate | Ox. CYP R125/I435    |
|                 |                        |                       | (Ahuja et al., 2013)    | (Ahuja et al., 2013)  |

(?? An extensive search for this set of proteins did not give conclusive results.).

transmembrane segments of CYP and CPR (Gideon et al., 2012; Srejber et al., 2018).

Our recent work on cytochrome P450 mechanism discusses the scope for interaction of CYPs with reductase and transferring electrons via protein-protein interactions (Parashar & Manoj, 2021). The topographies of diverse CYPs cannot afford any meaningful binding with the unique CPR. Further, mobility of these proteins is too low to afford kinetic viability for such a process. Instead, the accessibility of proximal cysteine to solvent and the direct activation of oxygen by CPR is strong evidence for murburn perspective in the endoplasmic reticular system. Moreover, the multi-molecular sequential scheme of the classical mechanism would have very little probabilistic viability, as compared to the stochastic bimolecular murburn scheme involving DROS. It is also important to note that the attempts to find purported direct protein-protein interactions between the CYP-CPR redox partners have failed under physiological conditions (Burris-Hiday & Scott, 2020). It was possible to simulate the reactions of diverse P450s like CYP2C9 and CYP2E1 with merely the respective CYPs + substrates + superoxide, without CPR and Cyt. b5 (Manoj et al., 2016b; Parashar et al., 2018). Also, depending on the reaction conditions, additives (like vitamin C or unrelated redox protein like Cyt. c) into reaction milieu could bring about favourable or deleterious outcomes in P450 reactions (Manoj et al., 2016b). This strongly suggested the involvement of DROS as electron-relay and catalytic agents.

3.5 Analysis of mechanism and probability of contact-interactions

For an effective interaction of Cyt. c with Complex III, the former must be oxidized whereas the binding subunit of the latter’s must be reduced. In the same milieu, the favorable binding of Complex IV with Cyt. c is quite the reverse scenario, that is- Cyt. c must be reduced and Complex IV module must be oxidized. So, the probability (based on the redox state of the components) of each interaction is 1/2 x 1/2 = 1/4 and the overall probability score for the sequential and directional outcome is: 1/4 x 1/4 = 1/16. The same logic applies for the ETs mediated by Cyt. b5 (from CPR to CYPs). Now, we have conclusively established that there is no topographically conserved binding-based logic between the diverse redox partners explored herein. In this context, Table 3 lists the purported ET transfer contact points between the affinity bound donor-acceptor ET complex that we could collect from literature. There is hardly any consensus on this aspect, as different groups report different sets of residues to aid affinity-based recognition. It is easily envisaged that the probability of getting any two proteins to interact through single point contacts is like finding needle in a haystack.

To highlight this, we explore one deterministic classical proposal, of a purportedly conserved arginine (R125) on CYPs serving as a bridge between the propionates of its own heme and the heme of Cyt. b5. Conservatively, there must be >50 positive surface residues on human CYP3A4 and there are >25 negatively charged surface residues on human Cyt. b5 (18 E and 8D, besides the heme propionate, as seen from Table 53, Item 3 of SI). Not all of these surface residues on the redox partners would enable effective ET; only one of the 50 positive residues (R125) on CYP and one of the 25 negative sites on Cyt. b5 is supposed to give an effective outcome. Therefore, this favorable probability (based on the redox state of the components) would be 1/50 x 1/25 = 1/1250. Of course, imposing dimensional constraints imposed by the two-dimensional lipid bilayer/membrane would change this score. But the overall probability of a favorable outcome is further a multiple of the probability scores of redox states and electrostatic interactions (1/16 x 1/1250), making such proposals a highly untenable scheme of events within realistic physiological dynamics! Our earlier works provide detailed aspects of probability analysis of classical purviews in steps/processes like Q-cycle (Manoj et al., 2020c) and Z-scheme (Manoj et al., 2020a). Now, considering the facts that- (a) R125 is not conserved in all CYPs (and neither is the purported alternative of I435) (Figure S1 of Item 5, SI), (b) It is common knowledge that Cyt. b5 is an optional component (and not obligatory) for effective ETs between CPR and several CYPs, (c) CYPs have diverse surface topographies (Parashar & Manoj, 2021; Srejber et al., 2018), (d) CYPs have their proximal cysteine ligand accessible to solvent (Parashar & Manoj, 2021; Venkatachalam et al., 2016) (e) ETs can occur between CPRs and cytochromes without physical contacts, when separated by dialysis membrane (Manoj et al., 2016a), (f) CYPs can carry out the physiological reaction with mere superoxide alone (Manoj et al., 2016b; Parashar et al., 2018), (g) Higher concentrations of Cyt. b5 is deleterious to reaction outcomes, (h) redox protein from one organism can work well with a partner from other organism (thereby downplaying the concept of co-evolution of partners), etc.- the classical proposal appears redundant and murburn proposal can be deemed relevant within the physiological realm. The murburn proposal’s viability is testified by the ubiquitous availability of various ions (like H+) and molecules/DROS (e.g. O2/¹O2−) in the milieu, which could effectively serve as fast...
agents of ET relay. The relative conservation of some amino acids in some instances (for example- abundance of lysines on the heme side of Cyt. c) may aid some aspects of this relay and is in no way an antise- nse to the m Rub-burn model.

It is known that the overall conservation of stoichiometry of amino acids is important in evolutionarily conserved proteins, from a structure-function correlation perspective (Burnett & Nguyen, 2011; Mittal et al., 2010, 2020). To further indulge the classical binding-based paradigm’s tenets (that positively/negatively charged residues of the cytochromes are responsible for identification and binding to the suitable surface of their diverse redox partners), we surveyed the statistical conservation of Arg, Asp, Glu, His, and Lys residues and the variations/deviations from average (and the data for the same is given in Item 6, SI). It can be seen that neither the actual percentage of these amino acids nor their deviation from average value lend any statistically coherent substantiation to the classical proposal.

While literature shows that electrostatically complementary amino acid residues are supposed to help determine affinity-based binding, the actual ET step is not supposed to need such requisites, as seen from Table 3. In this work, we have established that there is no conservation in surface topology or surface amino acid residues for ensuring an affinity-based binding logic across the organisms. Under the classical purview, this must imply that the ET process is mediated via just one-point contact, which would be very inefficient because of low probability of such a deterministic event. Even otherwise, how can the donor versus acceptor role of the cytochromes be differentiated? That is- if some lysines determine Cyt. c’ s binding to Complex III (when the former is oxidized), what is it that enables protein detachment after ET occurs and what is it that prevents binding with the same protein yet again? Further, why cannot the same lysines attach to some unfavorable locations on its redox partners, given that there is little evidence for affinity driven binding? Such considerations moot the binding-based ET as a low-probability event.

4. Conclusions
While the classical mechanism seeks inherent genetic conservation of amino acid residues at key positions (in the sequence) and in topology, the m Rub-burn ET logic does not pose such requisites. Conservation of some amino acids at a definite locus is not a conclusive support for the classical scheme and it does not counter the m Rub-burn ET logic either. Strong binding-based interactions and deterministic outcomes are characteristic of two- electron reactions with well-defined substrates. If binding-based long-distance ET via Marcus model was the rationale employed in the redox 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. From the comprehensive analysis conducted herein, we could not find conclusive evidence for affinity-based interactions driving ETs involving cytochrome c/b5. Since the purported key residues for binding are not necessarily conserved, the binding (even if demonstrated in vitro or in situ) would be inefficient and vary from protein to protein. These outcomes would have low physiological significance. Quite contrastingly, the kinetic/thermodynamic treatments indicate the involvement of DRS. The reaction milieus of Cyt. c/b5 compose the most fundamental routines of life and we suggest that ETs by these proteins may not necessitate affinity-based binding of the donor and acceptor pairs. We propose that proteins like Cyt. c and b5 are more likely to serve as non-specific electron relays/capacitors. This is an inference which justifies the promiscuity of interactions involving these proteins, evolutionary mandates and Ockham’s razor (principle of parsimony). The binding-based classical theory fails to explain the acute toxic effects of small amounts of small species like cyanide on ET outcomes in physiology. The intermediacy of small molecules, ions and DROS (m Rub-burn concept) explains the toxic effects of cyanide (Manoj et al., 2020f; Manoj & Soman, 2020) and interfacial uncouplers in the metabolic/physiological milieu (Manoj et al., 2019a, 2016b, 2019b, 2020c; Parashar et al., 2014; Parashar & Manoj, 2021).

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ORCID
Daniel Andrew Gideon http://orcid.org/0000-0003-2470-550X
Vijay Nirusimhan http://orcid.org/0000-0002-5567-0656
Jesu Castin E http://orcid.org/0000-0001-5019-0947
Karthik Sudarsha http://orcid.org/0000-0001-6299-5689
Kelath Murali Manoj http://orcid.org/0000-0003-4515-994X

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