Role of thylakoid membranes in oxygenic photosynthesis:

A comparative perspective using murburn concept

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Abstract: Murburn concept is a new redox metabolic paradigm which advocates that several redox enzymes generate/stabilize diffusible reactive (oxygen) species (DRS or DROS) to carry out useful electron/moiety transfer reactions at biological membrane interfaces (Manoj 2020a). Herein, we show that the components and principles of redox reactions within chloroplasts/cyanobacteria share several similarities with soluble and simple extracellular or peroxisomal heme-enzymes that carry out electron/group transfer. We explore the comparison in detail with membrane-embedded and complex systems that catalyze: (i) microsomal xenobiotic metabolism and (ii) mitochondrial oxidative phosphorylation. We point out that the murburn interpretations of catalytic phenomena are consistent through the various reaction systems cited above. Further, we argue that evolutionary constraints and the physiological restrictions of neutral pH ranges discount proton-gradient based explanations for bioenergetic phosphorylations in chloroplasts. Therefore, we propose that the highly packed thylakoid membranes with minute aqueous volumes serve to enhance the lifetimes of oxygen-centered radicals and intermediates. The murburn perspective could also potentially explain protein supercomplexes in chloroplasts, and generation of ATP in mitochondria by photo-activation. Our proposal also highlights the evolutionary significance of lipid membranes and utility of oxygen in diverse life processes.

Keywords: photolysis; photophosphorylation; chloroplast; oxidative phosphorylation; xenobiotic metabolism; photorespiration; murburn concept;

Introduction

Cells/organelles are defined and demarcated from their surroundings by the presence of lipid membranes. In both prokaryotic cells and eukaryotic photosynthetic organelles, lipid membranes form highly folded and convoluted structures to embed light-trapping redox/photo-active proteins/pigments. Stacks of thylakoid membranes (grana) form the hub of photosynthetic activity within chloroplasts. The major part of prevailing textbook explanations (Lehninger et al. 2004; Berg et al. 2002; Voet and Voet 2011) of membrane-interface phenomena of light reaction of photosynthesis (such as: Z-scheme of electron transport chain, Kok-Joliot cycle of oxygenesis, chemiosmotic rotary ATP synthesis or CRAS, Q-cycle, etc.) were formulated by 1970s. However, the structural details of chloroplasts and its various membrane-embedded components like photosystems (PS) and chlorophyll binding proteins/light harvesting complexes (CBP/LHC) were revealed later (Benson et al. 2015; Wei et al. 2016; Young et al. 2016; Mazor et al. 2017). Therefore, a revisitation of the structure-function correlation and mechanism of membrane-embedded photosynthetic machinery of chloroplasts is mandated.

Over the last two decades, our pursuits have highlighted how major redox enzymes could use DROS (diffusible reactive oxygen species) via murburn (*mur*ed *burn*ing; involves molecules-unbound ions-radicals interactions) concept (Manoj 2006; Manoj and Hager 2001, 2006, 2008; Manoj et al. 2010a, b; Andrew et al. 2011; Gade et al. 2012; Gideon et al. 2012; Parashar and Manoj 2012; Parashar et al. 2014a, b; Manoj et al. 2016a-d; Venkatachalam et al. 2016; Manoj 2017; Parashar et al. 2018; Manoj 2018a, b; Manoj et al. 2018; Gideon et al. 2019; Jacob and Manoj 2019; Manoj 2019; Manoj et al. 2019a-b; Manoj 2020a-c; Manoj and Manekkathodi 2020; Manoj and Soman 2020; Manoj et al. 2020 a-d). In particular, our proposals provide a "radical" rationale for understanding the orchestration of metabolic pathways in the vicinity of phospholipid membranes. A simplified representation of murburn interactions is shown in Figure 1. As shown, we advocate that O_2-DROS-H_2O equilibrium serves as a primary electron/moiety transfer catalytic agent in routine redox metabolism. This new perspective of electron/moiety transfer (murburn concept) has revamped key mechanistic underpinnings in hemeperoxidases (HPO), mixed function heme/flavin containing cytochromes/reductases of liver microsomal xenobiotic metabolism (or mXM), and the cascade of heme/flavin/Fe-S/Cu proteins of

mitochondrial/prokaryotic oxidative phosphorylation (m/p OxPhos). Such developments could also explain intriguing physiologies like biothermogenesis and hormetic/idiosyncratic dose-responses. We have also reported conclusive quantitative evidence/arguments to support the murburn explanation for cyanide (CN) toxicity in aerobic respiration, discounting the hemeFe binding-based classical rationale (Manoj and Soman 2020; Manoj et al. 2020a). In the context of this manuscript, it is known that CN and azide are inhibitors of multiple steps of photosynthesis (Berg and Krogman 1975; Nakatani 1983; Forti and Gerola 1977; Hill et al. 2014). Also, low-level laser therapy (LLLT) enhances mitochondrial ATP generation, which is also correlated to DROS production (Xu et al. 2008; Sommer et al. 2015; de Freitas and Hamblin 2016). Prima facie, these facts indicate a strong case for commonality of reaction mechanisms in mitochondria and chloroplasts, suggesting the relevance of murburn concept in oxygenic photosynthesis (Manoj and Bazhin 2019; Manoj 2020c; Manoj et al. 2020 a-d; Manoj and Manekkathodi 2020). Also, several structural and metabolic aspects of chloroplast machinery cannot be explained adequately by the classical explanations. Therefore, we explore membrane-based redox metabolic systems to see if murburn concept could afford a viable proposal for the light reaction occurring around the vicinity of thylakoid membranes of chloroplasts.

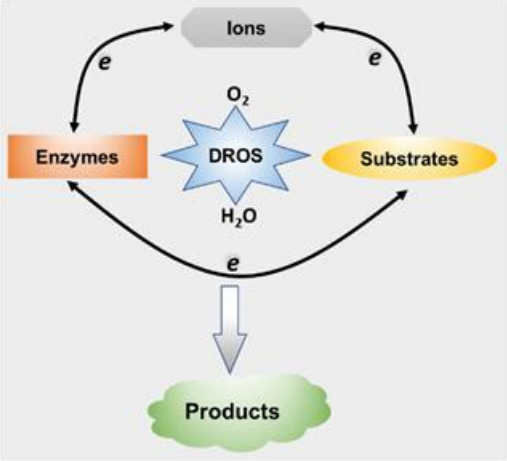

Figure 1: A simplified representation of the components of murburn concept, which invokes the involvement of diffusible reactive oxygen species (DROS), to relay electrons and transfer groups in redox enzymology.

1. Similarities in oxidative- and photo- phosphorylations in mitochondrial and chloroplasts

Earlier, we revamped the mechanism of mOxPhos system based on its mechanistic similarities with the mXM system (Manoj 2017). A similar exercise is undertaken herein by comparing mOxPhos with the photolysis-photophosphorylation (Pl-Pp) system, as shown in Figure 2.

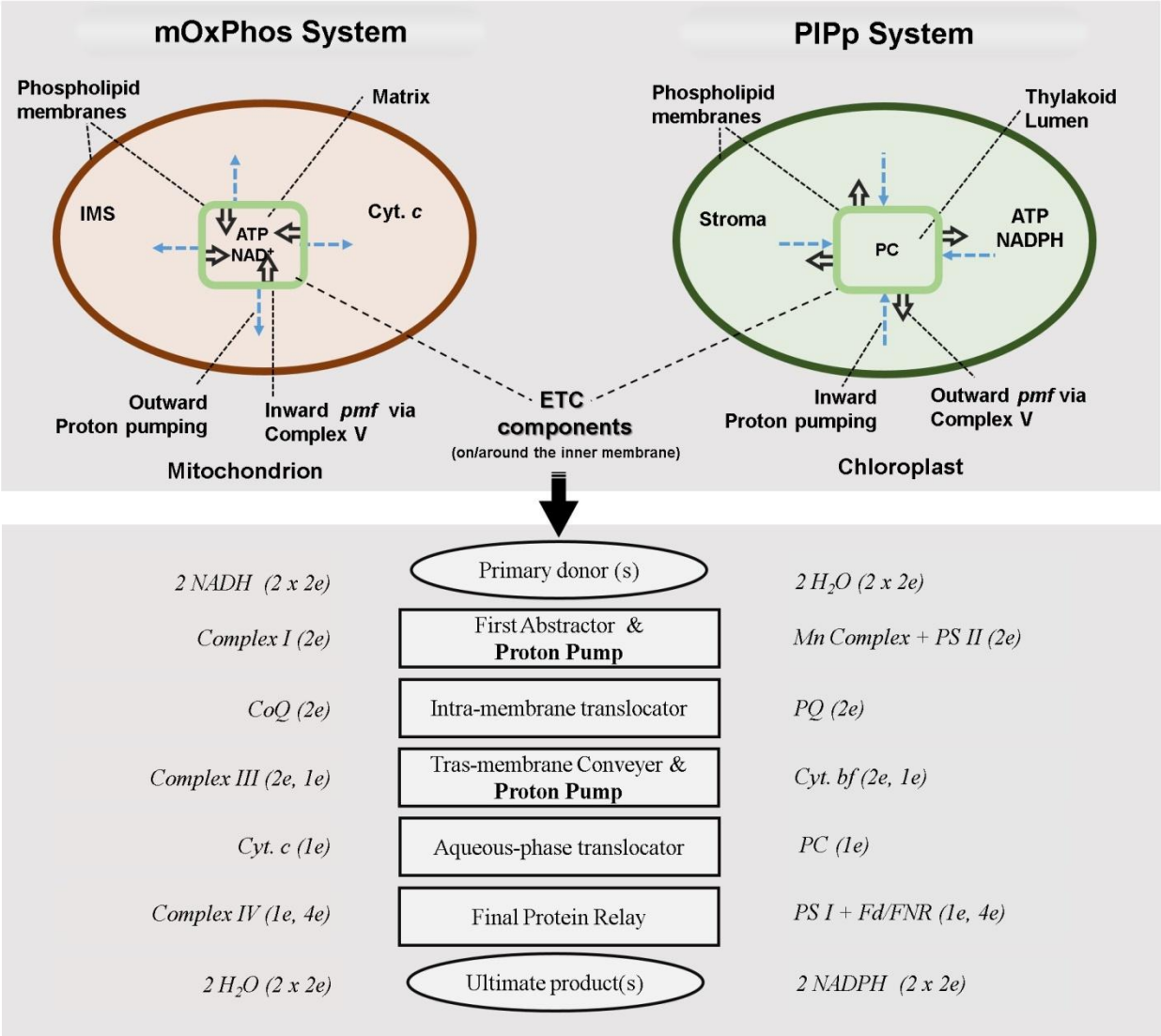

Figure 2: A comparison of structure-function correlations and mechanistic aspects of mOxPhos and Pl-Pp systems. The vectorial orientations of proton-pumps/Complex V and electron transport chain are depicted. The contents within the brackets signify the electron acceptance capacity and/or transferability of the given component. Both mOxPhos (mitochondria) and Pl-Pp (chloroplast) systems are also proficient generators of DROS and heat. As per the earlier perceptions, the elements of ETC were seen strategically located/connected energy transducing agents that aided in proton pumping across the inner membrane. The murburn model sees the ETC components as independent ADP-binding and DROS-modulating membrane-embedded agents that harness the catalytic utility of DROS at the membrane lipid and bulk aqueous interface (Manoj et al. 2019a, b).

The mOxPhos reactions occur at the inner membrane of mitochondria and are driven majorly by the chemical energy of NADH oxidation. Similarly, Pl-Pp occurs around the inner membrane of chloroplasts and is driven by radiant energy derived from sunlight. A quick survey of the elements of these two systems allows us to infer that mOxPhos and Pl-Pp could be deemed similar or anti-/quasi- parallel, because:

(i) In both systems,. micron-scaled organelles employ electron transfer phenomena at their inner phospholipid membrane interface, across distances of ~10^3 Å within time-frames of 10^3 s^{-1} per cycle. Also, both processes employ a series of multi-protein complexes with diverse redox centers (hemes, Fe-S proteins, flavins, etc.); and produce ROS and liberate heat during their functioning.

(ii) Both paradigms avail diverse redox systems for (supposedly) forming intricate electron transfer/transport chains (ETCs). The systems incorporate the common elements of proton pumps, membrane-based organo-quinone 2-electron translocator and aqueous-phase based metallo-protein 1-electron translocator.

(iii) Overall, the ETC reaction schemes in both systems require the input of a total of 4-electrons from two substrate molecules (two NADH/succinate molecules in mOxPhos and two water molecules in Pl-Pp) to give an output of two reduced molecules (two water molecules in mOxPhos and two NADPH molecules in photophos).

(iv) In both ETC systems, the first step purportedly involves the receipt of two/four-electron equivalents from the substrate(s). But before the transfer of the two-electron equivalents to the membrane-harbored quinones in the next step, several one-electron relays are supposedly involved in these highly complicated systems (Complex I in mOxPhos and Photosystem II in Pl-Pp).

(v) In the last step of the ETC, after receiving the one-electron equivalents from the aqueous-phase metallo-protein electron-translocators (Cyt *c* and plastocyanin), the final protein relays in both the systems are supposed to transfer electron pair equivalents to the ultimate products.

(vi) Very strikingly, the element of trans-membrane electron conveyor (called Complex III in mitochondria and Cyt *bf* in chloroplasts) is common, receiving 2-electron equivalents from membrane-quinones and giving away 1-electron equivalents to an aqueous phase.

Therefore, quite like the erstwhile perception of mOxPhos, electrons are supposed to be transferred through a series of membrane-embedded protein-bound redox centers as well as get transported via mobile carriers. The electronic pit-stops belong to various classes like cytochromes, quinones, hemes/chlorophylls and Fe-S proteins. The purpose of this whole exercise is to create a proton gradient across the membrane. Based on quantitative logic and available experimental data, the prevailing explanation of the “Rotary ATP synthesis - Chemiosmosis - Proton pump – Electron Transport Chain (RCPE) was questioned recently and

an evidence-based murburn scheme was proposed in lieu (Manoj/Manoj et al. 2017-2020). Though several stark differences exist between mOxPhos and Pl-Pp (with respect to structure/orientation of catalysts and partitioning of participants), the comparison above shows that deterministic multi-molecular reaction schemes and the production of DROS/heat are common to both systems. Also, if the fundamental charging of the Pl-Pp system with photons is discounted, the rest of the processes are all downhill reaction paradigms that could well fall within the purview of the murburn scheme.

2. Analyzing the cyanobacterial system for OxPhos and Pl-Pp

Unlike the eukaryotic plants that have both mitochondria and chloroplasts (two distinct organelles to carry out the separate electron flows of OxPhos and Pl-Pp respectively) within their cells, cyanobacteria conduct both photosynthesis (Pl-Pp) and respiration (OxPhos) within their thylakoids. They do so using the common machineries of Cyt. b_6f, plastoquinone and Cyt. c_6. In Figure 3, the simplified architecture of a cyanobacterium and the extant understanding of cyanobacterial respiratory and photosynthetic ETC are shown. It can be seen that maintaining the directionality of uphill and downhill electron transport in the two counter-directional ETCs is not possible simultaneously, as this would lead to futile cycles.

Figure 4A depicts the essential requirements of a controllable working model, which includes the efficient coupling of uphill and downhill machineries. In such a system, the elements of the uphill and downhill reactions are distinct and there are separate (inter-connected) reservoirs of the "starting fuel" synthesized or used. In Figure 4B, the currently prevailing mechanistic impression of the cyanobacterial metabolism is shown. As seen, this model has a susceptibility to drain (wastefully) with the given configuration. As soon as the light illumination stops, catabolic activities would drain the redox equivalents, killing the cell. This is better explained with an analogy from day to day life. A battery that is put on a load of "x" watts cannot get simultaneously charged by another capacitor of "y" watts ($y \approx x$), using the same electro-motive force conduits. An ordinary automobile's battery gets charged and takes up load at the same time owing to a modular setup where the power loop logics are distinctly demarcated. Even in these

setups, as soon as charging alternator is taken off, the system would be drained off all power, if the load remains connected.

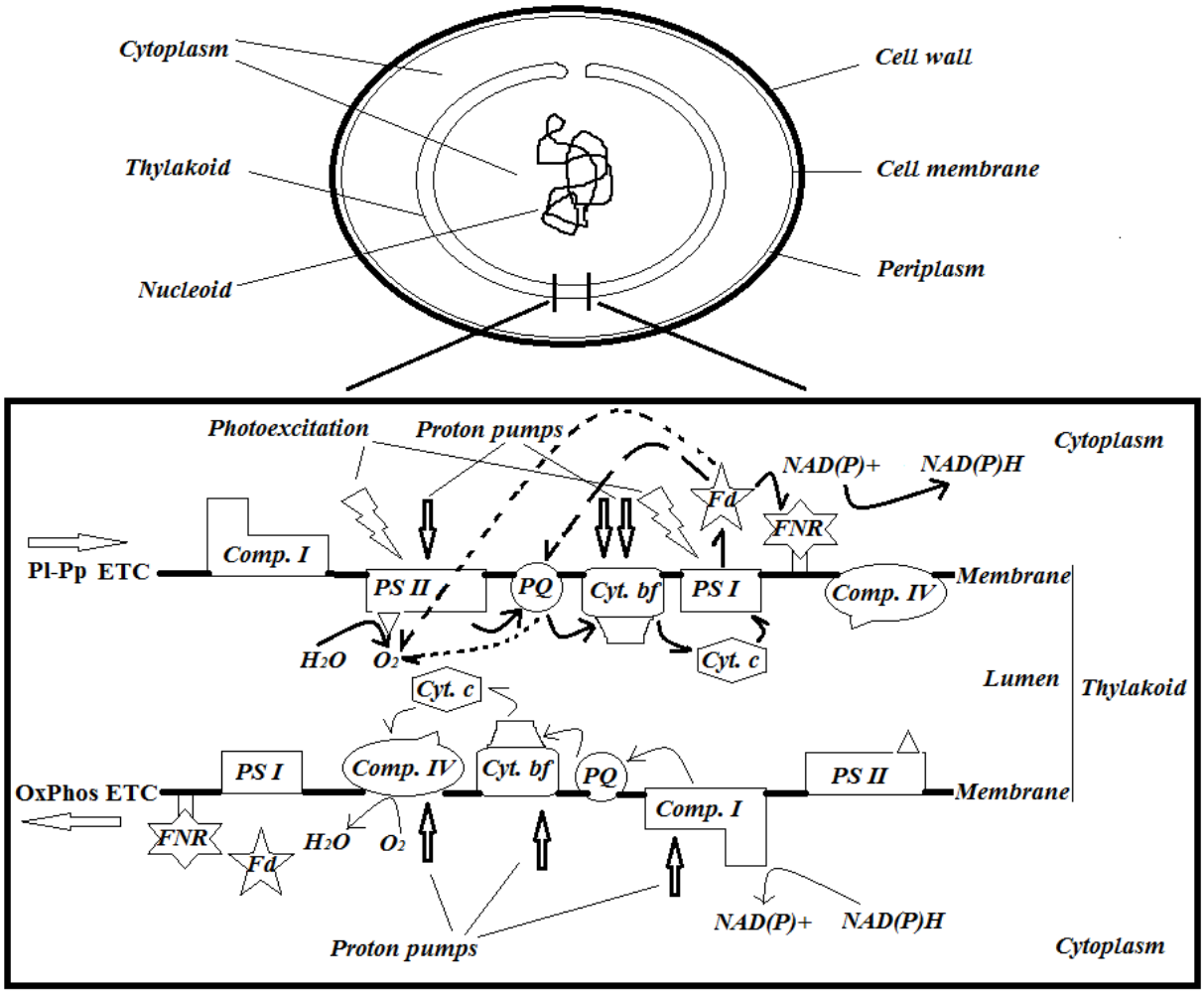

Figure 3: Schematic of cell structure and ETC proteins' distribution of thylakoid membranes of cyanobacteria*. Though there are little deterministic ways that the elements of the Pl-Pp or OxPhos ETC can be selectively or preferentially placed adjacently on a given membrane, we have presented the two systems' perceptions on the top and bottom membranes respectively.*

Under the prevailing perspective, it is difficult to see an unintelligent Cyt. c_6 deciding on imparting electrons to PS I or Complex IV. This "decision making" is crucial because it could lead to an "active synthesis" of NADPH or depletion of the same. That is- let us imagine that the bacterium is availing sunlight. Cyt c_6 would more probably give electrons to Complex IV (rather than a transient electron deficient PS I); and this Complex I would keep breaking down any NADPH that ever formed in the cytoplasm. The result would be that photoexcitation would only lead to a "cycling" of protons/water from thylakoid to cytoplasm. It is unlikely that a protein like

Cyt. c_6 can be instructed intelligently to switch between the OxPhos and Pl-Pp cycles, based on fluctuating cellular demands. The current mechanism seeks Cyt. c_6 to "drive up and down the road at the same time", around the membrane within anti-parallel traffics of OxPhos and Pl-Pp. Such a mechanistic demand of a deterministic ETC is destined to fail.

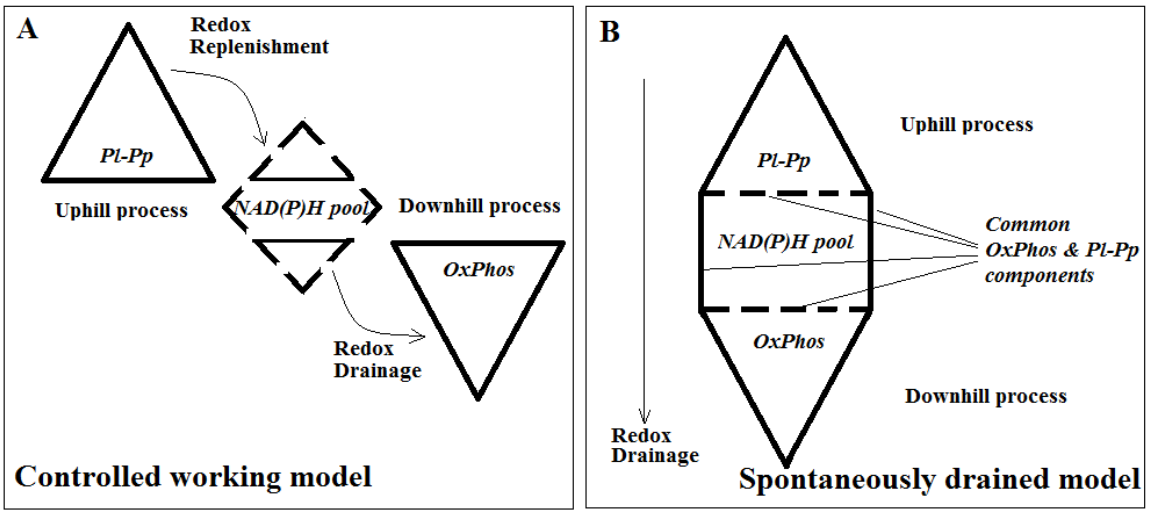

Figure 4: Comparison of controlled working model (A) and spontaneously drained model (B) schemes for cyanobacterial redox metabolism.

Several non-conventional but proven and accepted electron transfer possibilities exist in the Pl-Pp system, of which three putative interactions mediated by PQ (plastoquinone), Fd (ferredoxin) and FNR (ferredoxin-NADP reductase) are denoted by dashed connectives in Figure 3. Other components of the Pl-Pp system, namely- PS II, PS I, etc. could also give electron(s) to the omnipresent O_2 and DROS. If so, the currently perceived Pl-Pp ETC would be just "a wishfully deterministic scheme", and less of a probabilistically relevant pathway. This statement would be further explicated in the section below.

With the best possible orientations and juxtaposition of the proteins, there is yet a stark problem with the current explanations. That is- if proton pumps and a deterministic ETC were the key operatives, the very same Cyt. b_6f would be expected to pump a higher load of protons into the lumen, while indulging in its responsibilities for Pl-Pp cycle. This is because while the OxPhos cycle would have three proton pumping Complexes (including Complex III, the equivalent of Cyt. b_6f), the Pl-Pp machinery has only Cyt. b_6f serving the role of proton pump. We have

pointed out that this proton-pumping cannot be managed by the 'energy packet' available to Complex III (the mOxPhos equilvalent of Cyt. b_6f) (Manoj 2018b). Further, we see no justifiable purpose served by having a trans-membrane "electron-routing" step and self reduction of quinones (via the Q-cycle), all of which would only render the "ETC" kinetically non-viable with respect to the overall electron transfer rates. Recent developments in respirasome structures confirm this deduction, rendering the "quinone cycle" a redundant feature of the Mitchellian paradigm. For an exhaustive critique on the concept of ETC, please refer our recent works (Manoj/Manoj et al. 2017-2020).

Very importantly, the smallest photosynthetic cyanobacteria belong to the *Prochlorococcus* types, which have an average dimension of half a micron. Such a small organism would have practically no "free protons" within the cell to serve any proton pumps' requirements at physiological pH (Manoj 2017; 2018a, b). Therefore, under the premise that proton pumping in micron-dimensioned organelles is questionable, the current understanding would leave little modalities for ATP synthesis within the Pl-Pp paradigm and cannot explain why the electron needs to take such a roundabout route from water to NADPH either. Therefore, a study of the cyanobacterial mOxPhos & Pl-Pp ETC shows that the extant explanations for electron transfers and driving mechanisms for ATP synthesis are non-viable. On the other hand, existing features afford ample scope for the application of the non-deterministic murburn scheme in Pl-Pp.

3. The effects of additives: Uncoupling proteins of plants and molecules that "uncouple the useful reaction from the catalytic cycle" within mXM, mOxPhos & Pl-Pp systems

Uncoupling is a term used in redox enzymology when the enzymes use up the redox equivalents, but do not give fruitful reactions that are physiologically relevant. For example- in the mXM system, NADPH (the source of redox equivalents) could get used up without hydroxylating the xenobiotic substrate. Instead, DROS accumulation or water formation may happen. Similarly, in the mOxPhos system, NADH gets used for heat/DROS/water formation, without ATP synthesis.

Uncoupling proteins (UCP) in mitochondria cause heat generation and floral thermogenesis is well known in plants. Particularly, the skunk cabbage is famous for its ability to achieve even a

25 °C higher differential from a near-freezing ambiance (Ito-Inaba 2014). [A chloroplast-based heat generation process is also known, and this is attribted to non-photochemical quenching (NPQ) at higher light radiance. However, this is not the topic of discussion here.] The thermogenesis phenomenon was believed to result from a UCP-mediated uncoupling phenomenon, which supposedly led to the dissipation of trans-membrane proton potential (TMP) into heat. Based on new awareness, we have recently shown that UCP-mediated uncoupling and thermogenesis resulted due to its ability to sponsor DROS-based reaction chemistry around the inner mitochondrial membrane (Manoj et al. 2018). The extant explanation cannot clarify the beneficiary effect of UCP in photosynthesis (Sweetlove et al. 2006) whereas the murburn explanation offers scope.

In liver microsomes, the N-term peptide sequence of a flavoenzyme, cytochrome P450 reductase (CPR) was supposed to be involved in essential redox partnering with a heme-enzyme, cytochrome P450 (CYP). It was noted that when the N-term segment was truncated and left within the reaction medium, significant "uncoupling" resulted. We had shown that the uncoupling mediated by the N-term segment (deemed to be functionally analogous to UCP/dinitrophenol of mitochondria) resulted, owing to its ability to modulate DROS (Gideon et al. 2012). The reaction schemes of mXM and mOxPhos are compared with Pl-Pp in Figure 5.

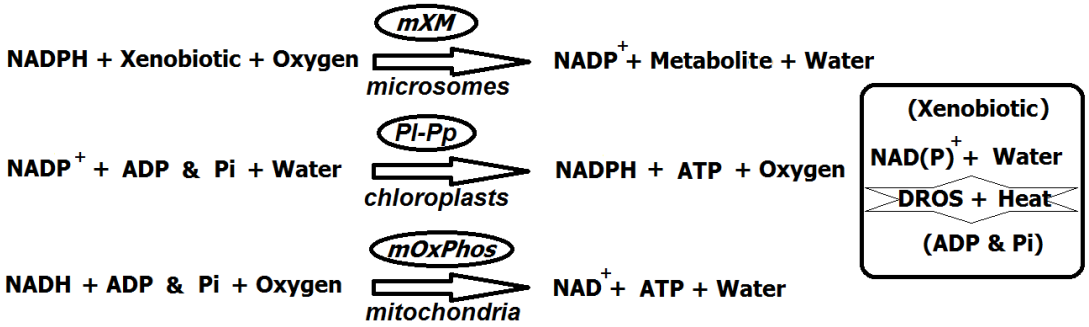

Figure 5: The similarities between the productive reactions and uncoupling phenomena observed in mXM, mOxPhos and Pl-Pp systems. *Uncoupled reaction outcome is given in the box on the right.*

It is seen that quite similar to the microsomal and mitochondrial systems, the chloroplast system also shows uncoupling reactions (giving heat and DROS), which were perceived to be due to "faulty electron transfers". Some molecules like disubstituted phenolics (like dinitrophenol or DNP, which is known to uncouple oxidative reactions with ATP-synthesis), are also known to

cause uncoupling in mXM and Pl-Pp systems, implying that the uncoupling mechanism is common across these membrane-embedded redox systems. Since the mXM system has no relevance for proton-pumps and since the proton-potential based explanation for mOxPhos system is untenable, the feasibility of DROS-based murburn scheme needs to be explored to explain the affects/effects observed in Pl-Pp. The new rationale proposes that DROS modulation at the membrane interface by molecules like DNP is facile due to interfacial catalysis mediated by extra-orgenellar protons (and not owing to dissipation of proton-gradients, as believed earlier). Further, all three systems (mXM, mOxPhos & Pl-Pp) are simple distributions of protein complexes on lipid membranes and Ockham's razor suggests that a simple stochastic theory is more plausible than a sophisticated deterministic theory. This inference is further corroborated with the results obtained by the introduction of additives as probe molecules within the reaction milieu. In such reductionist experimental strategies, the following results stand out-

(i) If Pl-Pp ETC only employs Marcus' outer sphere model of electron transfer, how can several man-made dyes and redox active molecules put in and draw out electrons from the purportedly deterministic ETC (Hauska 1977)?

(ii) The prevailing mechanistic understanding cannot account for the unusual concentration-dependent effects brought about by certain additives in Pl-Pp systems (Avron and Shavit 1965; Watling-Payne and Selwyn 1974). Such outcomes are typical manifestations of the operational relevance of murburn scheme involving radicals (Parashar et al. 2018).

(iii) With the Pl-Pp ETC, it is inexplicable as to why cyanide could serve as a toxic principle in the Pl-Pp process. Both Photosystems I and II (whose functioning is supposedly inhibited by cyanide) is not known to have any Fe-hemes and the inhibition is not owing to any binding-based effects, either. Emerson showed that the dark process is temperature sensitive but the light process is not (with respect to oxygen yield). At low temperature, 10 μM cyanide gave higher inhibition at short duration of darkness or continuous light, whereas it gave no inhibitory effect at longer dark periods. Such outcomes can be reasoned out within the murburn scheme, as cyanide modulates DROS (Parashar et al. 2014b; Manoj et al. 2016a, c; Manoj and Soman 2020; Manoj et al. 2020a).

(iv) Prasanna Mohanty's works (from Govindjee's group in the early 1970s) had shown that certain cations adversely affect the photosynthetic outcomes and we have argued that such outcomes result in redox metabolic schemes because the negatively charged superoxide would be liable to modulation at the phospholipid interface (Manoj et al. 2019a, b).

Considering the commonality of elements and the effects of diverse additive-generated outcomes, a simple chemical equilibrium/reaction scheme (like murburn concept) is a more likely explanation than an electro-mechanical proposal (like CRAS).

4. Comparing proton availability in cytoplasmic reticulae, mitochondria and chloroplasts

Since cytoplasm is practically an open source of water (as plasma membrane is not impervious to protons), protons are available for the membrane proteins (like cytochrome P450s) lodged on cytoplasmic reticulae. However, 3-dimensionally constrained micro-domains like mitochondria and chloroplasts are different. Gauging from electron micrographs, an average thylakoid is ~0.5 micron in linear dimension, and the lumen is ~0.05 micron thick. Therefore, assuming a flattened (cubic or cylindrical) vesicular structure, the maximal volume of a thylakoid would be $\sim 10^{-17}$ liters. As a result, in the starting or resting phase, the crude calculations for protons within the lumen of thylakoid a cellular physiological pH of 7 would equal $6.023 \times 10^{23} \times 10^{-7} \times 10^{-17} \approx 0.6$. More precisely, assuming that chloroplast have an average resting pH of 8 and thylakoids are stacked cuboids with a dimension of (1 x 0.015 x 0.233) microns (Austin and Staehlin 2011), the number of protons per lumen of an average thylakoid is = $6.023 \times 10^{23} \times 10^{-8} \times (1 \times 0.015 \times 0.233) \times 10^{-15} = 0.02$. Therefore, thylakoids are practically aprotic and this could enhance the stability of radicals significantly. (This does not mean that protons are not available for catalysis. Grotthuss mechanism would ensure catalytic process but pumping protons out into another and discontinuous macrosystem is a totally different thermodynamic process.) The stroma would have a much higher volume (say, 5 μm x 5 μm x 5 μm) of $\sim 10^{-13}$ liters, and it would have protons approaching = $6.023 \times 10^{23} \times 10^{-7} \times 10^{-13} \leq 10^{4}$. (It should be borne in mind that these differences in numbers cannot lead to gradients. This is because the proton concentration in the two phases are still the same, and therefore, a pH gradient cannot exist.) Assuming an initial

closed system of 100 thylakoids within a chloroplast (at say, time t1), only if the protein complexes efficiently pumped >90% of the stromal protons into the thylakoids (at say, time t2), the gradient across the thylakoid membrane would approach the requirement for ATP synthesis. This is given by the relation : 61 log {90/(0.5 x 0.5 x 0.05)}/{1000/(5 x 5 x 5)} = 180 mV. Here, the volume term of a thylakoid is ~0.01% of the stroma. Even if the thylakoids are connected, we would have the same calculation because while the total lumen volume increases by 100 folds, the proton amount in the lumen also gets multiplied by 100. It is known that 180 - 200 mV (correlating to a concentration gradient of $\sim 10^3$) is the minimal theoretical or practical requirement for deriving or observing ATP synthesis, per the "transmembrane pump" mechanistic purview (Nicholls 2004). The calculation above is from an initial state to a final state and it cannot explain the steady state functioning of the chloroplast, quite like the scenario of mitochondria (Manoj 2017, 2018b). Since the pumps are not synchronized and there are no modularities (that is- a protein pump is not associated with its own "outer covering shell", and several such shells are not triggered simultaneously with some phase transition operation), the proton concentration would rather equilibrate in steady state, than create a gradient. Such simple calculations challenge the "proton pump" concept for the thylakoid membrane and generate even more questions. Why should there be thousands of proteins to deal with a small number of protons? Also, how can the proteins flip back and forth into different conformations under a steady state trans-membrane potential, which they themselves set up? Quite simply, in the absence of any direct evidence for proton pumps, all these impossible requirements seek too much intelligence at the proteins' ends. [A survey of textbooks and reviews shows various mechanisms and citing different numbers of protons per each of the complexes. For example: (i) while Ahern shows PQ transferring protons to the lumenal side, Lodish depicts Cyt. b_6f carrying out the same function and (ii) while Ahern does not see PS II as a proton pump at all, Lehninger deems PS II as a trans-membrane pump (pumping protons from stroma to lumen) and Lodish considers PS II a proton abstractor from stromal phase. Peter Mitchell (who proposed mitochondrial-membrane proton pumps) did not deem Complex IV of mitochondria a proton pump while it is considered one nowadays. *For editors' and peer reviewers' perusal, a supplementary information file is attached presenting a visual snapshot of several such discordances in literature.*] Such a scenario questions the theory and experimental crux of the proton pump concept. We believe that thermodynamics does not support assumptions that the

protons would “have any urge or energy” to spontaneously return to the stroma (Manoj 2017). For further discussions in this regard, please refer the follow-through communication on our critique of the RCPE explanation for mOxPhos (Manoj 2018b).

5. Explorative queries based in evolutionary perspectives and structure-function correlations

The following queries and analyses raise significant concerns that enable us to think beyond the available paradigms and consider alternative approaches for explaining oxygenic photosynthesis.

(i) Since the CRAS mechanism is questionable, the elaborate ETC scheme of Pl-Pp would have little evolutionary or functional significance. That is- why would nature evolve such an ETC that doesn’t serve any significant function? Or more importantly, how could the ETC evolve, as none of the proteins can function on its own towards a fruitful outcome. Other queries are also relevant, for example: why does the PS II not relay the electrons from water directly to Fd/FNR?

(ii) All “evolutionary logic” arguments stated against the RCPE explanation (like “non-evolvability due to irreducible complexity” for mOxPhos (Manoj 2017; Manoj 2018b) are also relevant for Pl-Pp. A vitally deterministic scheme could not have evolved downplaying the inherent reactivity of the components constituting the system. Why should the cellular system choose a lesser efficient system when substrate-level oxidation gives more efficiency (and that too, without the threat of forming DROS)? It is difficult to envisage how Complex V could function reversibly *in situ* (with the same mechanism for forward and backward reaction) when it has higher affinities for ATP, in comparison to ADP (Manoj 2018b; Manoj et al. 2019a, b).

(iv) Why do leaves which show high rates of photosynthesis also have low life spans (Matsuiki and Koike 2006)? This reality would be highly counter-intuitive and counter-evolutionary, if RCPE principles were operative.

(v) The available tructural information available leaves little scope for a modularized synchronization or trans-membrane potential (TMP) tapping mechanisms for the various structure-function components to work in tandem, with respect to the RCPE explanation. The

chloroplast is an oxygenic system. Oxygen (singlet/triplet) and DROS are spontaneously formed in chloroplasts. It is difficult to imagine that any electronic circuitry or redox catalytic/relay mechanism could evolve without oxygen/DROS destabilizing the "vitally deterministic mechanisms" (Manoj 2017; Manoj 2018b). Structural elucidations have not demonstrated any specific oxygen evolution routes within PS II for a "safe-release" outside the chloroplast. The fact that most PS II systems are seated deep within the thylakoid stacks is antithetical to the hitherto attributed structure-function correlations. This is because PQ would have to jump through several stories of thylakoid membranes to relay electrons from PS II to the peripherally located Cyt. b_6f.

(vi) Chloroplasts have varying numbers of grana and stories/stacks of thylakoids within grana (as seen in Figure 6), with a random distribution of the thylakoid proteins and pigments, often showing significant variations based on the particular species, locations within the leaf, durations of development, etc. Such an arrangement not speak of a "deterministic vital scheme" that the CRAS explanation solicits. Particularly- (a) From the PS II centers located deep within the multi-layered stacks of thylakoids, it is inconceivable how the Cyt. b_6f - plastocyanin system could relay an electron in any feasible time to the outer stack-located PSI centers for explaining the "Emerson effect". (b) With the current model, the deep-seated PSII systems cannot work either in conjunction with the PSI center (in a "non-concerted" setup where PSI is blocked, say), or independently. Surely, it would be even more difficult to get the electrons relayed to the Ferredoxin(Fd)-NADP reductase (FNR) system present within the stroma. So, why should there be multi-storied stacks of thylakoids (when it is easier in an evolutionary sense, to have one-three layers, at the most, for efficient electron transfers between the various Complexes)?

(vii) The distances between the redox centers and distributions of the elements cannot achieve deterministic or viable electron transfers. The structures of PS I and PS II (for example: Fromme et al. 2001; Young et al. 2016; Nelson and Ben-Shem 2004; Nelson and Yocum 2006) show that many times, the distances between key redox centers are above 12 Å (the crucial distance that could give a viable electron transfer rate with respect to the overall timescales observed physiologically). Further, there are several other redox active species that are located (much closer by) within transferable distance. If Marcus theory was the fundamental operative logic, it

is quite unclear as to why the closer located species could not shunt the proposed ETC routes. The arrangement of redox potentials are also not in the strict ascending gradient, as exemplified by the F_A to F_B purported transfer in PS I leg of the ETC (Manoj et al. 2020d). The logic used against the "vitally deterministic ETC of mOxPhos (Manoj 2018b) is relevant for the Pl-Pp system too.

6. Conceptualizing a minimalist murburn paradigm for the light reaction of photosynthesis

In mitochondria, online measuring shows direct evidence that ATP synthesis is correlated to DROS concentration (Nicholls 2004; van Hameren et al. 2019). The production of DROS was sidelined earlier as side-reactions or pathophysiologies but we have recently shown that ATP synthesis via DROS is the viable physiological route (Manoj et al. 2019a, b). Photo-therapy in medicine (LLLT, as discussed in the introduction) is explained by several theoretical assumptions (Zielke 2014; de Freitas and Hamblin 2016; Sommer 2019). We believe that the most chemico-physically sound and parsimonious mechanism invokes obligatory roles for DROS. Support for our theory is available by a work which shows that incorporation of light-harvesting chlorophyll pigments also allow mitochondria of animals such as *Caenorhabditis* and mammals to produce ATP upon photo-activation cues (Xu et al. 2014). Surely, it is a misplaced idea now to consider that DROS are purely deleterious, as a lot of direct beneficial evidence of DROS-assisted metabolism and signaling has begun to accrue since the last couple of decades (Schulz et al. 2007; Heidler et al. 2010; Mittler 2017). Yet, a discerning individual may ask- isn't murburn concept a rather chaotic reaction mechanism, as it uses diffusible radicals? To this, we say that murburn principle is best illustrated via a simple analogy: A cloth daubed in water first and oil later can be set on fire. Though this is a highly chaotic exothermic reaction, the fabric does not get charred significantly so long as there is oil to burn. Similarly, as long as there is a sustained supply of metabolic substrates present that are dynamically reacted and removed as products, the scaffold of lipid membrane is left more or less intact. (Of course, the membrane does get affected deleteriously cumulatively, and this is one of the reasons for aging and death.) In this regard, we would like to reproduce (verbatim) the concluding section of an article from our group (Jacob and Manoj 2019)-

"Just because a cut-injury in the kitchen most probably results from knife-abuse, one does not infer that knives do not have any positive role in the kitchen! Also, one may find gloves and cutting-boards to hold

and handle knives in the kitchen. Their presence too does not imply that knives have no constructive culinary roles! (In this analogy- knives, gloves and cutting boards are equivalent to DROS, redox enzymes and antioxidants respectively.)"

Having addressed the aestheic concern, we carry out a reductionist analysis of some redox metabolic systems and routines first, to piece together a better physiological understanding of their roles later on. In this regard, we present Table 1 for elucidating the commonalities of reaction components of select redox catalytic systems, as we go from a simple system to a more complex one, from left to right columns.

Table 1: Compositional attributes of redox enzymatic systems. *The various systems show significant similarities in the formative elements of the reaction systems. It is likely that systems with common components abide by similar mechanisms. Text in large braces (within items 1 & 2) are examples cited for the proteins.*

Item	Extracellular	Peroxisomal	Microsomal	Mitochondrial	Cyanobacterial	Chloroplastid
1. Primary coenzyme	Porphyrin [Haloperoxidase]	Porphyrin [Cat., Perox.]	Porphyrin [CYP]	Porphyrins [Comp. III, IV]	Porphyrins [Comp. IV/PS II]	Porphyrins [PS II]
2. Secondary coenzyme	(Mn^{2+})	(NADH)	Flavins [CPR]	Flavins [Comp. I, II]	Flavins/Carotenoids [Comp. I/CBP]	Carotenoids [LHC, CBP]
3. Tertiary component	Nil	GSH, Ascorbate	Cyt. b_5, Adx (Fe-S)	CoQ, Cyt. *c*, Fe-S	PQ, Cyt. c_6, Fdx (Fe-s)	PQ, PC, Fdx (Fe-S)
4. Membrane	None	None	One, open	Two, closed	Two/several, closed	Several, closed
5. Activators	H_2O_2/$^*O_2^-$	H_2O_2/$^*O_2^-$	NAD(P)H + O_2	NAD(P)H + O_2	NAD(P)H + O_2 + hv	NAD(P)H + O_2 + hv
6. Substrates	RH	RH	RH	ADP + Pi	NAD(P)H/$NADP^+$ + ADP + Pi + H_2O (OH^-) (+ O_2)	$NADP^+$ + ADP + Pi + H_2O (OH^-) (+ O_2)
7. Ions	H^+, X^-	Nil	OH^-	H^+, Mg^{2+}	H^+, OH^-, Mg^{2+}	H^+, OH^-, Mg^{2+}
8. Mediators	DROS	DROS	DROS	DROS	DROS	DROS
9. Products	H_2O + RX	H_2O + R-R	H_2O + H_2O_2 + ROH	ATP + H_2O + H_2O_2	ATP + H_2O + H_2O_2 + NAD(PH) + O_2	ATP + H_2O + H_2O_2 + NADPH + O_2

Though there are notable differences in composition, it can also be noted that there is a fundamental framework of similarity across the systems. Figure 6 shows the locations of various redox metabolic systems explored in this study- both within and outside the cell. CYPs are not just found within cytoplasmic endoplasmic reticulum (microsomes), they are also localized in mitochondria (Sangar et al. 2010) and chloroplasts (Warzecha 2016) too. Similarly, catalase/peroxidases are not only found in vacuoles, cytoplasm or periplasm; their presence was also deomonstrated in mitochondria and chloroplasts (Prasad et al. 1995; Arita et al. 2006; Heinze and Gerhart 2002). Since it is beyond reasonable doubt that the hemeproteins use diverse DROS for catalysis and we have consolidated the murburn theory in mitochondria too, it is forthright to deduce that the presence of such diverse redox proteins in chloroplasts is owing to

the evolutionary significance of DROS-based metabolism across diverse organelles and organisms.

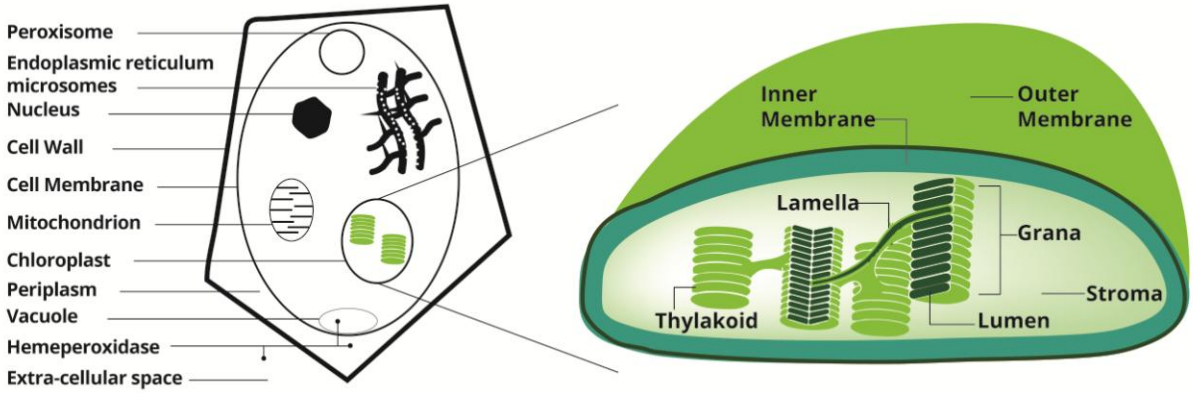

Figure 6: The localization of various redox enzymatic systems compared within this study (not drawn to scale) and the structure of thylakoid membranes' stacking into grana within chloroplasts.

Therefore, we hypothesize that DROS are the common elements across these diverse systems and the presence of one-electron redox-active centers enable an efficient stabilization/utilization of radical species like superoxide. Further, we propose that since oxygen is more soluble in lipids and protons have lower availability in the immediate vicinity of lipid environment, embedding redox proteins enables further efficiency in the redox processes which harness DROS. The lipid stacking is even more dense (than what is observed in mitochondria) and the lumen of thylakoids is constrained to minimize free water and protons, so as to achieve a greater density of protein/pigment/DROS loads, to enhance the efficiency of photosynthesis (which involves hydroxyl radical). Light falling on various pigments could enable electron emission but only PS I and PS II could enable efficient charge separation. Other chlorophyll and carotenoid pigments within LHCs and CBPs could generate superoxide, which would serve as electron donors, but would regenerate oxygen. Therefore, the light absorbed and electrons emitted by miscellaneous LHCs/CBPs/pigments would not show up in the overall tally and only electrons availed from water would give a directional reaction. A brief sketch of the proposed murburn model is shown in Figure 7. As in other systems before, the reaction components have multiple roles; water, oxygen and DROS are produced and utilized at multiple sites. For example- (i) water is used by PS II, and produced in ATP synthesis and by DROS reacting among themselves. (ii) superoxide could be produced and used at multiple loci like FNR, NDH (NADPH dehydrogenase),

CBP/LHC, PS I & II, etc. Since radicals are produced and released in the vicinity of membrane-embedded proteins containing redox centers, it makes bioenergetic sense to have a clumping of such complexes, to better harness the released species. If protein-protein binding was the actual mechanistic route, such homo and hetero complexes would only minimize the partner recognition ability. This was one of the major rationales that helped explain the existence of supercomplexes (respirasomes) in mitochondria (Manoj et al., 2019a). Our proposal is supported by the fact that photo-chemically released electrons around the thylakoid membranes would react instantly with oxygen to give superoxide. This reaction has a transformed Gibb's energy o term, $\Delta G^{o'}$ = -249.6 kJ/mol (Manoj & Bazhin, 2019). Therefore, the reaction equilibrium constant, K_{eq} = 10^{44} and the reaction rate constant, k = 2 x 10^{10} $M^{-1}s^{-1}$ (Takahashi et al., 1988), making our proposal thermodynamically and kinetically facile.

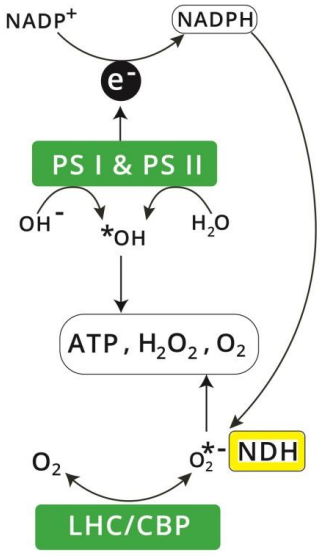

Figure 7: A brief outlay of the salient murburn scheme of reactions. *The large bulky complexes of PS I & II and NDH are immobilized on the thylakoid membranes to harness the DROS species reactivity and electron relaying capacity. While effective charge separation is achieved by PS I & II, CBP/LHC serve in keeping the reaction running with a steady pool of superoxide, which serves as effective electron relay agent to replenish 'electron holes'. (Fd, PQ and PC are considered charge capacitors in the murburn scheme; giving and taking electrons within the oxygen-superoxide equilibrium.)*

We present Table 2, which demarcates the perceptional change from classical views to murburn concept. The comparison within this table also highlights the inability of classical view to- (i) address key aspects of the mechanistic chemistry and (ii) capture the holistic picture of the discussions made with greater details and clarity. It must be noted that while the murburn

explanation is consistent throughout the systems, the classical purviews invoke different principles (at times, even 'fantastic' ideas like membrane potential sponsoring phospho-ester bond formation) across the diverse systems. The final queries of assessment (A-E, at the bottom of Table 2) enables the discerning reader to select the more suitable hypothesis. The extant paradigm of Pl-Pp requires the membranes to be highly regulatory and also seeks the membrane-embedded proteins to change conformations and make phospho-ester bonds based on mechanical cues (which is unheard and unseen, other than in CRAS mechanism). The murburn explanation for photosynthesis is a simple scheme of bimolecular chemical reactions that does not seek electro-mechanical intelligence for the chloroplast/thylakoid membranes. We have demonstrated the viability for DROS to make the phospho-ester bond and also provided thermodynamic and kinetic foundations to our proposals (Manoj et al. 2019a, b; Manoj and Bazhin 2019; Manoj et al. 2020a, b). Also, our theory is the only existing proposal that justifies the obligatory requirement and role for oxygen in diverse life forms.

Table 2: Erstwhile consensus perceptions vs. recent murburn attributions and other agendas of compariso: *For a given criterion, the upper(shaded) row depicts the classical view and the lower row is the murburn perspective.*

Criteria	Extracellular	Peroxisomal	Microsomal	Mitochondrial	Cyanobacterial	Chloroplastid
1. Energetic drive	Redox gradient	Redox gradient	Redox gradient	Redox gradient	Redox gradient	Redox gradient
	Thermodynamic pull	Thermodynamic pull	Thermodynamic pull	Thermodynamic pull	Thermodynamic pull	Thermodynamic pull
2. Reaction site	Enzyme pocket	Enzyme pocket	Enzyme pocket	Enzyme pocket	Enzyme pocket	Enzyme pocket
	Delocalized	Delocalized	Delocalized	Delocalized	Delocalized	Delocalized
3. Key species	Compd. I	Compd. I	Compd. I	Compd. I	Compd. I/S1-S4	S1-4
	Diverse/DROS	Diverse/DROS	Diverse/DROS	Diverse/DROS	Diverse/DROS	Diverse/DROS
4. Electron Transfers	Specific and affinity-based	Specific and affinity-based	Specific and affinity-based	Specific and affinity-based	Specific and affinity-based	Specific and affinity-based
	Non-specific, equilibrium-based	Non-specific, equilibrium-based	Non-specific, equilibrium-based	Non-specific, equilibrium-based	Non-specific, equilibrium-based	Non-specific, equilibrium-based
5. Primary e-Source	H_2O_2	H_2O_2	NADPH	NADH	NAD(P)H/H_2O	NAD(PH)/H_2O
	NAD(P)H/DROS	NAD(P)H/DROS	NAD(P)H/DROS	NAD(P)H/DROS	NAD(P)H/DROS/ H_2O	NAD(P)H/DROS/ H_2O
6. Final e-Sink	Products	Products	O_2	O_2	O_2/$NADP^+$	$NADP^+$
	O-H bond	O-H bond	O-H bond	O-H bond	O-H bond	O-H bond
7. Protons-Proteins	Active site relay	Active site relay	Active site relay	ETC-Proton pumps	ETC-Proton pumps	ETC-Proton pumps
	Murburn equilibriums	Murburn equilibriums	Murburn equilibriums	Murburn equilibriums	Murburn equilibriums	Murburn equilibriums
8. Membrane	NA, soluble enzyme	Not needed, soluble enzyme	Required to make CYP+CPR complex	Impermeable to H^+	Impermeable to H^+	Impermeable to H^+
	NA, soluble enzyme	Not needed, soluble enzyme	Enhances CYP-CPR synergy	Low permeability to H^+	Low permeability to H^+	Low permeability to H^+
9. TMP	NA	NA	NA	TMP drives ATP synthesis	TMP drives ATP synthesis	TMP drives ATP synthesis
	NA	NA	NA	TMP results from ATP synthesis	TMP results from ATP synthesis	TMP results from ATP synthesis
10. Kinetics	Classical (E-S interactions)	Classical (E-S interactions)	Classical (E-S interactions)	Classical (Quinone movements in	Classical (Quinone movements in	Classical (Quinone movements in

(limitation)				lipid phase)	lipid phase)	lipid phase)
	Atypical (Conditional)	Atypical (Conditional)	Atypical (Conditional)	Atypical (Conditional)	Atypical (Conditional)	Atypical (Conditional)
11. Q/PC/Cyt. b_5-c-c_6	NA	NA	Specific relay	Specific relay	Specific relay	Specific relay
	NA	NA	Generic capacitor	Generic capacitor	Generic capacitor	Generic capacitor
12. DROS/ Radicals	Toxic waste	Toxic waste	Toxic waste	Toxic waste	Toxic waste	Toxic waste
	Promiscuous mainstay	Promiscuous mainstay	Promiscuous mainstay	Promiscuous mainstay	Promiscuous mainstay	Promiscuous mainstay
13. Inhibitions	Active site	Active site	Active site	Block ETC or Proton pumps	Block ETC or Proton pumps	Block ETC or Proton pumps
	Active site or Milieu based	Active site or Milieu based	Active site or Milieu based	Active site or Milieu based	Active site or Milieu based	Active site or Milieu based
14. Uncoupling	Active site	Active site	Active site	Trans-memb. H^+ shuttling	Trans-memb. H^+ shuttling	Trans-memb. H^+ shuttling
	Milieu based	Milieu based	Milieu based	Milieu based	Milieu based	Milieu based
15. Dose response	Simple	Simple	Simple	Simple	Simple	Simple
	Simple/ Complex	Simple/ Complex	Simple/ Complex	Simple/ Complex	Simple/ Complex	Simple/ Complex
16. Heat prodn.	Water formation	Water formation	Water formation	TMP dissipation	TMP dissipation	TMP dissipation
	DROS quenching	DROS quenching	DROS quenching	DROS quenching	DROS quenching	DROS quenching
17. Transition state	Multimolecular	Multimolecular	Multimolecular	Multimolecular	Multimolecular	Multimolecular
	Bimolecular	Bimolecular	Bimolecular	Bimolecular	Bimolecular	Bimolecular
18. Networking of components	Serial, ordered	Serial, ordered	Serial, ordered	Serial, ordered	Serial, ordered	Serial, ordered
	Parallel, unordered	Parallel, unordered	Parallel, unordered	Parallel, unordered	Parallel, Unordered	Parallel, unordered
19. Stoichiometry	Defined	Defined	Defined	Defined	Defined	Defined
	Variable, non-integral	Variable, non-integral	Variable, non-integral	Variable, non-integral	Variable, non-integral	Variable, non-integral
20. Overall scheme	Deterministic	Deterministic	Deterministic	Deterministic	Deterministic	Deterministic
	Stochastic/ Statistical	Stochastic/ Statistical	Stochastic/ Statistical	Stochastic/ Statistical	Stochastic/ Statistical	Stochastic/ Statistical
A. Structures?	**No**	**No**	**No**	**No**	**No**	**No**
	Yes	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
B. Distribution?	NA	NA	**No**	**No**	**No**	**No**
	NA	NA	**Yes**	**Yes**	**Yes**	**Yes**
C. Promiscuity?	**No**	**No**	**No**	**No**	**No**	**No**
	Yes	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**
D. Evolvable?	NA	NA	No	No	No	No
	NA	NA	Yes	Yes	Yes	Yes
E. Ockham's cut?	No	No	No	No	No	No
	Yes	Yes	Yes	Yes	Yes	Yes

A to E: Queries whether the two purviews explain: structures of proteins/organelle, distribution of proteins and reaction components, promiscuity (of electron/group transfers, substrate preferences, etc.), appear to be irreducibly complex (ref. Michael Behe) and thus, non-evolvable, and finally- whether the hypothesis appeals to Ockham's razor (principle of parsimony).

Conclusions

Biological phospholipid membranes serve as anchoring platforms for redox bioenergetic or xenobiotic metabolic proteins. By virtue of low proton availability and higher O_2/ROS solubility within the lipid phase, the micro-dimensioned aqueous milieu ensconsed by phospholipid membranes aid metabolism by enabling the localization/stabilization of O_2/ROS in its vicinity. This is perhaps the reason that- (i) lipids with relatively low functionalization (as compared to other biopolymers like carbohydrates or proteins) became the evolutionary choice for cellular

envelope, (ii) high amounts of one-electron redox-active centers are found within the xenobiotic/bioenergetic metabolic systems' membrane proteins, (iii) membrane-embedded mitochondrial/chloroplast proteins aggregate to form (super)complexes to better scavenge or harness radicals released in each other's vicinity, and (iv) lipid oxidation/peroxidation is observed in physiology as a result of collateral outcomes. In the chloroplasts, practically random distribution of several one-electron active pigments and redox centers are noted. The light reaction (photosynthetic) rates are known to go up with the provision of substrates. There is significant relaxation in electron transfer specificities. Uncoupling is seen to occur with several molecules and unusual concentration-dependent effects of additives are noted. These are also seen in the mXM and mOxPhos systems, where murburn principles are well-explored and the presence and relevance of radicals are established. In the light of the fact that photosynthetic microbes like *Prochlorococcus* (cyanobacteirum) would not have free protons to serve proton pumps, it is natural to imagine that the murburn logic of electron and phosphate group transfers hold good in chloroplasts too. We believe that this new awareness can be used to avail better and more detailed explanations of the Pl-Pp process.

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