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Acute toxicity of cyanide in aerobic respiration: Theoretical and experimental support for murburn explanation

Abstract: The inefficiency of cyanide/HCN (CN) binding with heme proteins (under physiological regimes) is demonstrated with an assessment of thermodynamics, kinetics, and inhibition constants. The acute onset of toxicity and CN's mg/Kg LD_{50} (μM lethal concentration) suggests that the classical hemeFe binding-based inhibition rationale is untenable to account for the toxicity of CN. In vitro mechanistic probing of CN-mediated inhibition of hemeFe reductionist systems was explored as a murburn model for mitochondrial oxidative phosphorylation (mOxPhos). The effect of CN in haloperoxidase catalyzed chlorine moiety transfer to small organics was considered as an analogous probe for phosphate group transfer in mOxPhos. Similarly, inclusion of CN in peroxidase-catalase mediated one-electron oxidation of small organics was used to explore electron transfer outcomes in mOxPhos, leading to water formation. The free energy correlations from a Hammett study and IC_{50}/Hill slopes analyses and comparison with ligands ($CO/H_2S/N_3^-$) provide insights into the involvement of diffusible radicals and proton-equilibriums, explaining analogous outcomes in mOxPhos chemistry. Further, we demonstrate that superoxide (diffusible reactive oxygen species, DROS) enables in vitro ATP synthesis from ADP+phosphate, and show that this reaction is inhibited by CN. Therefore, practically instantaneous CN ion-radical interactions with DROS in matrix catalytically disrupt mOxPhos, explaining the acute lethal effect of CN.

Introduction

Since the discovery of the dye Prussian blue (ferric ferrocyanide, $Fe_7[CN]_{18}$) and the volatile prussic acid (hydrogen cyanide, HCN) about three centuries ago, cyanide salts and acid (hereon termed CN) have captivated the attention of scientists, commoners and social authorities. Owing to its acute lethality, CN is relevant in cases of suicide/homicide, and therefore, this chemical's availability is restricted [1]. As shown in Scheme 1 (top panel), cyanide (CN) toxicity is traditionally understood to result from its ligation to hemeFe centers of vital proteins like mitochondrial cytochrome *c* oxidase (COX) [2]. This stoichiometric and competitive event (where oxygen and CN bind to the same hemeFe locus) is supposed to prevent oxygen accessibility/transport/utilization by heme proteins. However, we believe that this view cannot account for the acute toxicity shown by low amounts of CN. Our works in the field question several long-standing beliefs on the mechanism of CN's role in heme enzyme milieu [3-5]. Further, based on the mechanistic insights derived from an extensive study of microsomal xenobiotic metabolic (mXM) system [6-12], we had critiqued the

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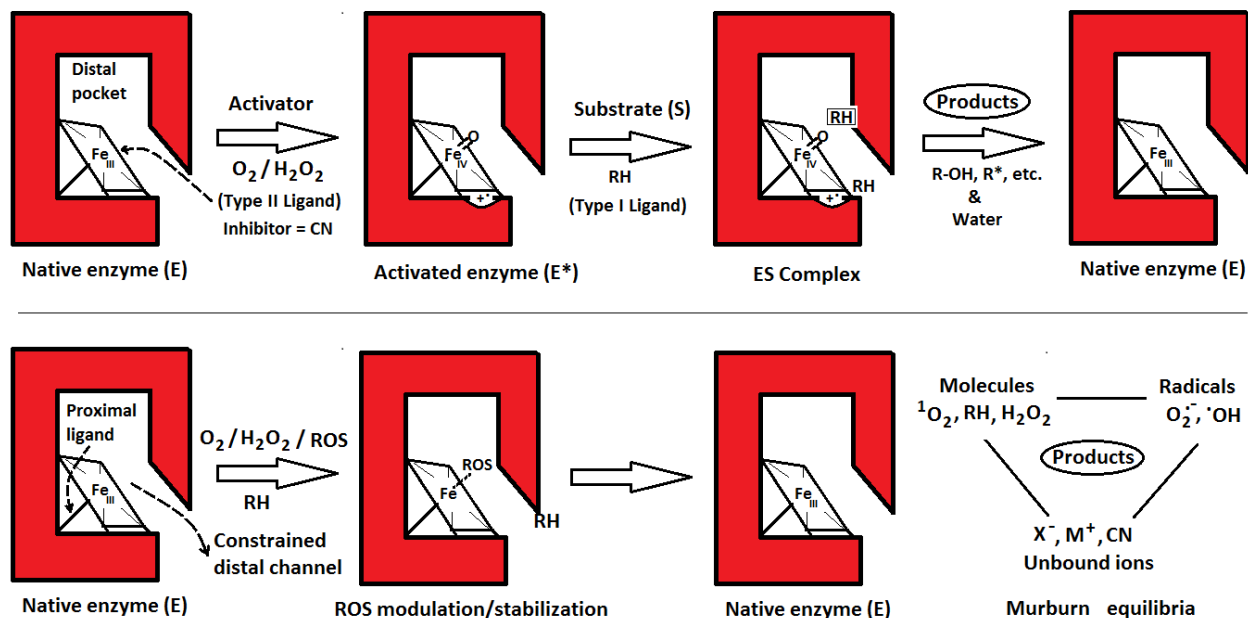
Scheme 1: The serial/sequential events in the classical scheme and the stochastic paradigm of reactions in murburn model of heme enzymes. In the classical mechanism (top panel), an oxygen atom from the activating source binds via Type II modality at the distal hemeFe (the same mode/locus as CN binding). This could be prior (heme peroxidase) or post (cytochrome P450) to Type I substrate (RH in box) binding (a high affinity process) at the same distal pocket, which occurs without direct interaction with hemeFe. Later, a two electron deficient Compound I is formed, which works by a proton/electron abstraction via heme edge interaction and/or oxygen-rebound at the heme-center. All substrates must access the heme center or edge for the reaction to occur in this fashion, and for the same, sophisticated switches are supposed to function within the proteins. Finally, this enzyme activity (resulting from an activated enzyme + substrate complex) leads to formation of various products, their detachment from the active site and regeneration of the native enzyme. In the expanded murburn scheme (bottom panel), the distal hemeFe pocket is primarily seen as a ROS generator/modulator/stabilizer, and the proximal ligand is seen as an electron relaying facet (enabling Fe redox equilibration). The apoprotein could also aid a low-affinity binding/presentation of the substrate RH, when the ROS emerges from the heme center. There could be a complex scheme of interaction of molecules and ions (in the vicinity of a few angstroms or nanometers of the protein), by virtue of the radical species involved, and this gives an array of products, with variable and non-integral stoichiometry. This scheme could explain the reports of several types of 'activated enzyme species' and diverse substrate 'preferences'.

currently held explanations for mitochondrial oxidative phosphorylation (mOxPhos, aerobic respiration) and championed a murburn concept based mechanistic paradigm for this fundamental routine of life [13-20]. This radical reaction perspective for oxygen utilization (murburn concept) proposes that diffusible reactive oxygen species (DROS) generated at the mitochondrial membrane-matrix interface catalyze ATP-synthesis, and CN ion-radicals non-competitively disrupt this DROS-mediated 'oxidative phosphorylation', via a catalytic mechanism (Scheme 1, bottom panel). Herein, we delve deeper into the issue with a reductionist experimental approach, to further elucidate the mechanistic underpinnings of CN toxicity in heme enzymology, and correlate it to the outcomes of aerobic respiration.

Before proceeding any further, we must quote Aldous Huxley (*"However elegant and memorable, brevity can never, in the nature of things, do justice to all the facts of a complex situation."*) and stress on the need for using simpler in vitro systems to understand the complex physiological system, as well as justify the models we employ in our current study. Mitochondrial metabolism is complicated owing to: (i) two lipid bilayer membranes ensconcing the reaction microenvironment, (ii) the involvement of multitudes of protein catalysts in various conformations and distributions, each with a number of redox cofactors, (iii) several substrates that could interact with various components within the tri-phasic reaction medium (thereby leading to a bevy of interactive equilibriums), (iv) the dynamic/steady state transports occurring within the system, and (v) presence of several transient and unstable reactive intermediates. CN is known to interact with hemeFe centers of vital heme proteins, particularly, COX, a key part of the mOxPhos machinery.

COX is a complex membrane-embedded dimeric protein with hemeFe and Cu cofactors, and several doubts linger on its role/control within the mOxPhos system [21, 22]. Therefore, we take here simpler/analogous models and study CN's interaction in more facile and directly 'monitored' hemeFe activity signatures. In such simple systems, the hemeFe and its ligand's (like oxygen or CN) binding interactions can be theorized minimally, as given in Scheme 2.

If CN binding is to heme-porphyrin systems is efficient (as is currently believed to be), we envisaged that the interaction of the distal heme-Fe pocket with different Type II ligands and Type I substrates would be reflected in the experimental outcomes. Availing the insights from such an approach could help us better understand similar/analogous fate of events in the mOxPhos system. The top panel of Scheme 1 shows details of the classical heme enzyme mechanism, which is fundamentally based on an activated enzyme making an ES complex (also, as in Scheme 2). Herein, all steps of catalysis transpire within the distal heme pocket. Over the last two decades, we have stressed on the limitations posed by the heme enzyme distal heme pocket in binding/catalysis and highlighted concentration-based outcomes and diffusible species mediated catalysis. New insights on utilization/interaction with activators (H_2O_2, organic hydroperoxides, $NAD(P)H+O_2$, etc.), diverse (pseudo)substrates and modulating additive molecules and unbound ions were presented [3-12, 23-31]. Via such reductionist and in situ explorations, the following simple key elements of murburn concept have emerged in complex heme-flavin enzymology: flavins activate molecular oxygen, penta-ligated heme-proteins stabilize and modulate the diffusible reactive oxygen species (ROS), and one-electron redox-active hexaligated heme-proteins (cytochrome c/b_5) or Fe-S proteins serve as non-specific electronic relays/buffers in the reaction milieu. It is to be noted that murburn concept does not negate the classical theory, but is a superior/larger paradigm that extends beyond the limits/limitations of the active site (distal pocket). At low heme enzymes/substrates and/or activators & inhibitors, a murburn interactive equilibrium (involving various redox active molecules, unbound ions and diffusible radicals) forms the products (including water) (bottom panel, Scheme 1).

Classically, one-electron or two-electron transfer/oxidation reactions mediated by heme-enzymes were supposed to result from hydrogen atom abstraction by direct interaction of the substrate with the catalytic distal Fe-O species or heme-edge and invoked an oxygen rebound mechanism. The currently perceived catalytic cycle routes through Compound I, an iron-oxygen porphyrin cation radical species ($Fe-O^{3+}$), which is thought to be involved in hemeperoxidases (HPOs), catalases, cytochrome P450s (CYPs) and COX [32-37]. The in vitro synthetic, spectroscopic, and crystallographic procedures use tens to hundreds of micromolar enzyme and tens to hundreds of millimolar substrate, many times in 'homogenized' single phase systems. In such high ligand:enzyme ratios and at high absolute enzyme concentrations, Compound I formation and substrate positioning at the distal heme-pocket becomes more probable. Only in such scenarios, the fastidious classical scheme requiring sequential and ordered formation of ternary complexes (with high affinity binding of substrates) becomes facile. After substrate binding and formation of the reactive intermediate at the heme center, the substrate must acquire a bonding distance with the hemeFe. Such a scheme would entail high enantioselectivity, must also show exceptional substrate preferences, and relatively occluded/inaccessible parts of substrates would not be attacked by the enzyme. However, the premises and outcomes in physiology and within the common in vitro assays for most heme enzymes do not meet the fastidious criteria required for Compound I mediated catalysis.

While "outside the active site" one-electron oxidations of pyrogallol/TMPD and two-electron moiety transfers of chlorination (of say, indene → indene chlorohydrin reaction) can be assayed within minutes at 10^{-10} to 10^{-8} M HPO [26-28], the active-site Compound I mediated oxygen atom insertions need 10^{-7} to 10^{-5} M levels of the same enzyme (for the same substrate, indene → indene oxide reaction) for effective catalysis [23,25,27]. It is known from experimental research that small Type II ligands bind to various heme enzymes at k_{on} values of 10^3 to 10^7 $M^{-1}s^{-1}$ (determined under highly optimal conditions, in vitro), over a wide range of pH [38, 39,40]. It is only forthright to deduce that the Type I binding by larger molecules would be slower or within the same ranges. Chloroperoxidase (CPO) mediated bleaching of diverse molecules (a 2e⁻ chlorine moiety transfer) and HPOs (like horseradish peroxidase (HRP) or CPO) mediated 1e⁻ oxidation of a molecule like 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) show overall pseudo-first order rates >10^2 s^{-1} [4,5,27,28]. This is when the heme enzyme is taken at <10^{-8} M and (pseudo)substrate is taken at 10^{-4} to 10^{-5} M, while the outcome is seen even for active-site excluded molecules, and in the absence of special evolutionary mandates for high affinity binding. Such a catalytic outcome is inexplicable with a purely active site process, as 10^7 $M^{-1}s^{-1}$ (highest rate for catalytic site access) x 10^{-8} M (active site concentration) = 10^{-1} s^{-1}. Further, we
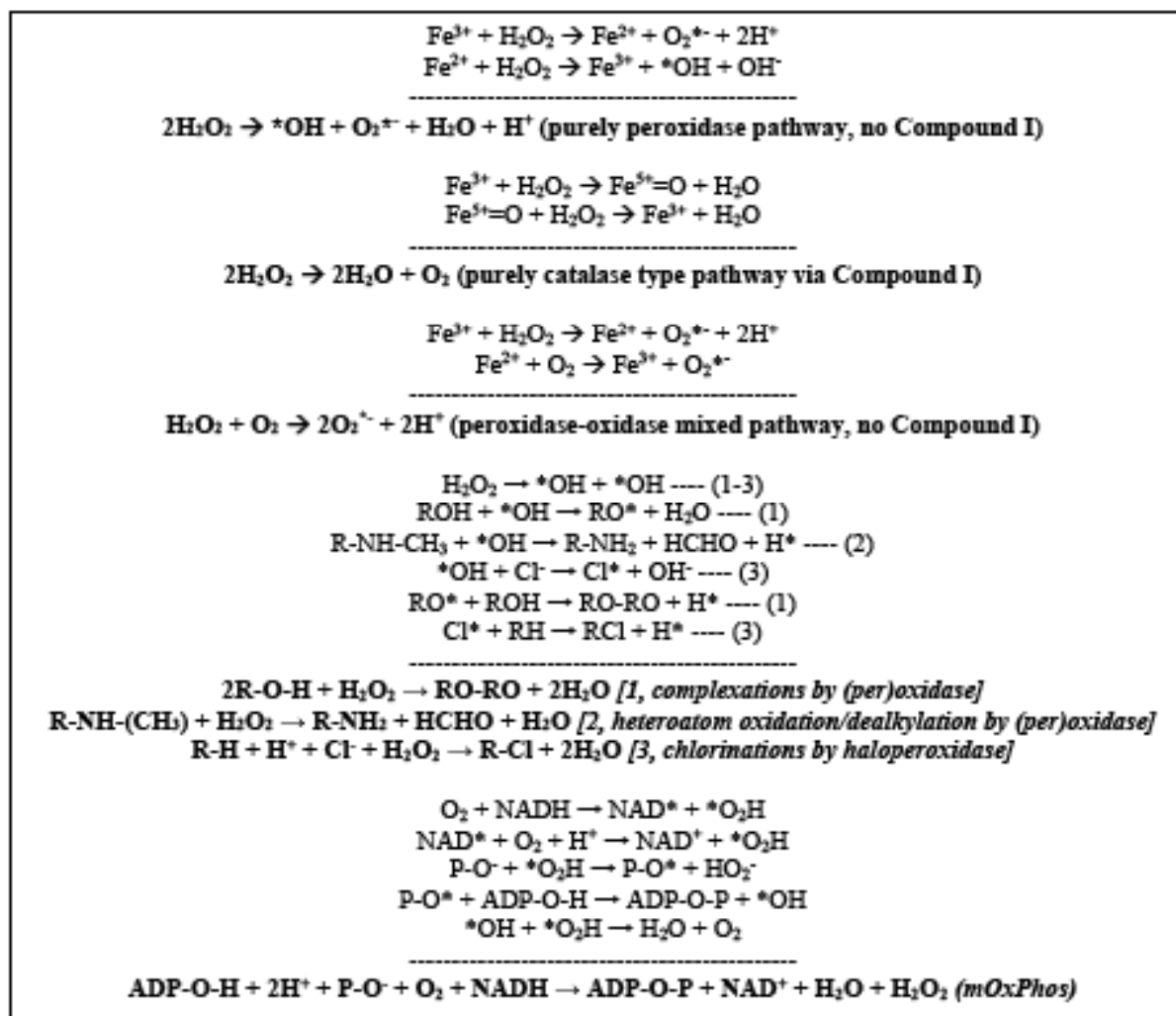
have shown that in such low enzyme/substrate mixtures and concentration ranges, binding of small ligands to heme enzyme is inefficient [3, 9]. Furthermore, in such scenarios, in the absence of one-electron facile oxidizable substrate, even a small activator molecule like H_2O_2 is not used by enzymes like CPO and HRP [4]. The redox equivalents from peroxide are used only in the presence of final substrate, thereby pointing to the presence of an inherent "thermodynamic pull" switch within the reaction milieu, and this does not need complex functionalities within the enzyme (like opening up of helical loops, etc.) [4, 11]. A mOxPhos enzyme like COX is embedded in lipid membranes (restricting multi-directional freedom of access) and the deep-seated distal hemeFe is accessible only via constrained channel(s). In membrane proteins-based activity assays, pre-incubation with the various proteins/components enhances reaction efficiency [11], which means that such proteins take more time to move/orient/juxtapose favourably in the membrane environment, in relation to the overall reaction kinetics. Therefore, mere 'affinity-binding at active site' based or 'protein-complexation mediated outer sphere electron transfer' based treatments cannot explain reaction outcomes and kinetics in such complex systems.

In the mXM system involving cytochrome P450 (CYP) and its reductase (CPR), we had resorted to a reductionist methodology to correct long-standing erroneous function attributions [3, 9, 11]. The prevailing perceptions that heme enzyme activated molecular oxygen and CPR pumped electrons to CYP via long distance electron tunnelling in a CPR+CYP+substrate+O_2 multimeric complex were corrected to show that CPR activated molecular oxygen to give DROS, which was stabilized and modulated by CYPs. The mOxPhos system is several orders more complex and the functional attributions in this system are even more obscure. Therefore, in order to minimize complications, we obviate the flavin chemistry with NADH+O_2 (which requires a four electronic reaction to give water, per the classical scheme!) completely and employ only one enzyme and the initial activator molecule of H_2O_2. Scheme 3 shows the various reactions investigated in the current study. Besides, cyanide, amitrol and azide are also used as Type II binders in diverse heme enzyme systems, as exemplified by fungal heme-thiolate (CPO), bovine heme-tyrosylate (catalase) and plant heme-imidazolate (HRP). For assessing the effect of CN on electron transfer reactions, TMPD (a classical substrate in COX assay) and pyrogallol (a classical peroxidase substrate) are taken as one-electron oxidizable molecules, concomitantly leading to water formation. To explore the mechanism of anion/moiety transfer, we traced the bleaching of thionine,
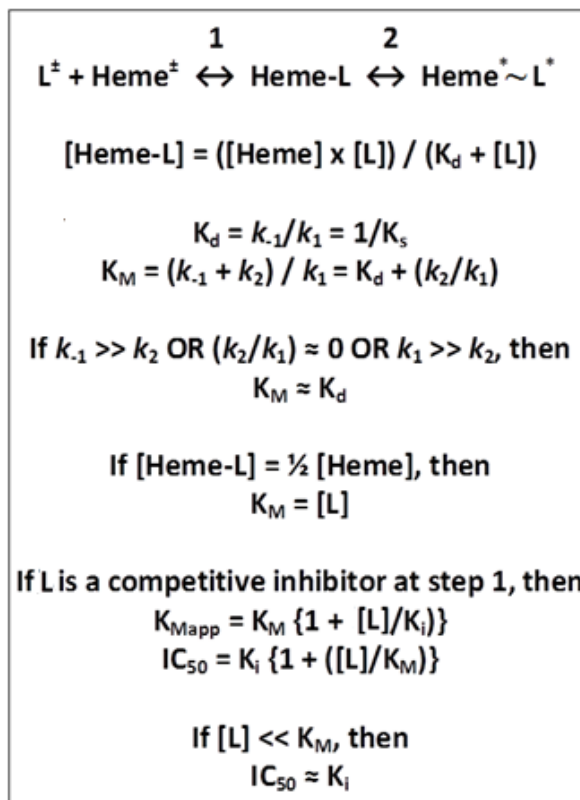
Scheme 2: Theoretical basis of equilibrium/kinetic constants for heme-ligand binding. The superscript of * and ‡ connote a change in charge/electronic states from the initial state. For example, heme can have Fe(II) or Fe(III) states and the ligand could be O_2 or O_2^{*-}, respectively. The original heme concentration taken is [Heme], the formed complex is [Heme-L] and the free ligand in milieu is [L]. Then, for [Heme] < [L] (that is, the ligand in milieu is in far excess of the heme protein) and assuming a 1:1 stoichiometry of Heme:L binding (with a defined site on the heme protein), the laws of equilibrium and steady-state approximations afford the correlations (the famous Hill / Michaelis-Menten / Cheng-Prusoff equations). The italicized small letter k is the pertinent rate constant (with units of s^{-1} or $M^{-1}s^{-1}$, depending on whether the reaction is first order or second order) whereas the capital K is the relevant equilibrium constant (in M). The forward reaction rate constants are k_1 and k_2 whereas the backward rate constants are k_{-1} and k_{-2} (the latter, k_{-2}, is deemed irrelevant for the current discussion now). The first step is the equilibrium interaction characterizing the K_d (dissociation constant) or K_s (stability constant). The second step is a chemical reaction, which plays a significant factor in the determination of the value of the functional interaction constant K_M.

thiourea and substituted styrenes in CPO's chlorination mixture. We demonstrate overwhelming commonalities (and some peculiarities) in such diverse simplified systems and a complex system such as mOxPhos, to better explain mOxPhos chemistry and physiology.

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + O_2^{\bullet-} + 2H^+$$
$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + {}^\bullet OH + OH^-$$

$$2H_2O_2 \rightarrow {}^\bullet OH + O_2^{\bullet-} + H_2O + H^+ \text{ (purely peroxidase pathway, no Compound I)}$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{5+}{=}O + H_2O$$
$$Fe^{5+}{=}O + H_2O_2 \rightarrow Fe^{3+} + H_2O$$

$$2H_2O_2 \rightarrow 2H_2O + O_2 \text{ (purely catalase type pathway via Compound I)}$$

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + O_2^{\bullet-} + 2H^+$$
$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\bullet-}$$

$$H_2O_2 + O_2 \rightarrow 2O_2^{\bullet-} + 2H^+ \text{ (peroxidase-oxidase mixed pathway, no Compound I)}$$

$$H_2O_2 \rightarrow {}^\bullet OH + {}^\bullet OH \text{ ---- (1-3)}$$
$$ROH + {}^\bullet OH \rightarrow RO^\bullet + H_2O \text{ ---- (1)}$$
$$R\text{-}NH\text{-}CH_3 + {}^\bullet OH \rightarrow R\text{-}NH_2 + HCHO + H^\bullet \text{ ---- (2)}$$
$${}^\bullet OH + Cl^- \rightarrow Cl^\bullet + OH^- \text{ ---- (3)}$$
$$RO^\bullet + ROH \rightarrow RO\text{-}RO + H^\bullet \text{ ---- (1)}$$
$$Cl^\bullet + RH \rightarrow RCl + H^\bullet \text{ ---- (3)}$$

$$2R\text{-}O\text{-}H + H_2O_2 \rightarrow RO\text{-}RO + 2H_2O \text{ [1, complexations by (per)oxidase]}$$
$$R\text{-}NH\text{-}(CH_3) + H_2O_2 \rightarrow R\text{-}NH_2 + HCHO + H_2O \text{ [2, heteroatom oxidation/dealkylation by (per)oxidase]}$$
$$R\text{-}H + H^+ + Cl^- + H_2O_2 \rightarrow R\text{-}Cl + 2H_2O \text{ [3, chlorinations by haloperoxidase]}$$

$$O_2 + NADH \rightarrow NAD^\bullet + {}^\bullet O_2H$$
$$NAD^\bullet + O_2 + H^+ \rightarrow NAD^+ + {}^\bullet O_2H$$
$$P\text{-}O^- + {}^\bullet O_2H \rightarrow P\text{-}O^\bullet + HO_2^-$$
$$P\text{-}O^\bullet + ADP\text{-}O\text{-}H \rightarrow ADP\text{-}O\text{-}P + {}^\bullet OH$$
$${}^\bullet OH + {}^\bullet O_2H \rightarrow H_2O + O_2$$

$$ADP\text{-}O\text{-}H + 2H^+ + P\text{-}O^- + O_2 + NADH \rightarrow ADP\text{-}O\text{-}P + NAD^+ + H_2O + H_2O_2 \text{ (mOxPhos)}$$

Scheme 3: A minimalistic mass and charge balanced representation/breakdown of heme-enzyme catalyzed reactions employed/studied in this work. Formation of water is seen in all reactions and all mechanisms involve the obligatory involvement of ROS and reactive radicals, and most reactions are essentially via a one-electron scheme. Adherence to this scheme would enable facile perturbation of outcomes by the addition of select redox-active additives, like CN. Further, high rates of reactions enable facile catalysis even by nanomolar levels of enzymes. In the current study, the complexation/dealkylation reaction has been employed to trace the influence of proton-availability and dynamics in CN inhibitions within heme-enzyme catalyzed electron transfer reactions. The chlorination reaction has been used for exploring linear free energy relations, the effect of substrate size, environment, etc. and also to afford a comparison with other Type II ligands' influence under functional scenarios of heme enzyme mediated moiety transfers. Though peroxidase mediated complexation, dealkylation and chlorination reactions have been shown to start from peroxide and involve the hydroxyl radical, the actual reaction can more efficiently go through an oxidase mixed pathway, which would involve superoxide radicals. This is the more favorable route at low concentration of heme enzymes, owing to the availability of oxygen and stability of superoxide in aprotic milieu. The reduced hemeFe can bind to an oxygen molecule, giving superoxide, which leaches out to react with the substrate, subsequently giving peroxide (as seen in the mOxPhos reaction). The probability of diverse reaction routes and multiple interactive equilibriums' involvement give variable stoichiometry and unusual dose response outcomes. Therefore, the stoichiometries shown in the reactions are not the only valid outcomes, and they are merely given for basic comprehension. Actual reaction stoichiometry and sequence/species may vary.

Materials and Methods

Cyanide's impact on IC_{50} and 'functional K_d' estimation in peroxidase-catalase systems: Horseradish peroxidase (HRP) and bovine catalase (product numbers P-6782 and C-9322 respectively) were procured from Sigma Chemicals, USA. Chloroperoxidase (CPO) employed for these studies was a pure preparation from Lowell Hager's (University of Illinois, U-C) lab. Other chemicals were purchased from reputed providers and were of analytical grade. The

details of enzyme assays and overall workflow have been published in detail earlier elsewhere [3, 4, 26-28] and other details are given in the pertinent figure legends or data discussion. Herein, only salient and very relevant specific details are given. Reactions were carried out at 27 °C in disposable cuvettes, and the concentrations of components are given in the particular figure legends. Statistically viable and verified data were used to plot and analyze the data for Hill slopes and IC_{50} values using GraphPad Prism 5.02. The structures of some ligands and (pseudo)substrates employed in this study are given in Scheme 4.

Free energy correlation for substituted styrenes' reaction in the haloperoxidase mixture and a comparison with simpler chlorinating agents/ environments: Suitable amounts of a commercially available sodium hypochlorite solution and chlorine gas were dispensed in buffer and a laboratory preparation of Cl_2O was used as 'control' chlorinating agents (with at least one chlorine atom in positive oxidation state). Using 100 μM solution of various para- and meta- substituted styrenes (at 2.5% CH_3CN), the maximal absorbance wavelength at pH 2.75 and molar extinction coefficients (as determined from the OD of the 100 μM solution, from which the OD of the above solution bleached with excess hypochlorite or subjected to CPO reaction until completion of reaction) at the respective λ_{max} given below, in Table 1.

Cyanide's impact on chlorine moiety transfers in chloroperoxidase activity and comparison with other Type II ligands: Conversion of (pseudo)substrates thionin and thiourea, in the presence of amitrol, cyanide and azide, was monitored with UV-Vis spectrophotometry, as per standard protocols [26-28]. The reaction was carried out in open quartz cuvettes and the final volume of the reaction mixture was 1.0 ml. 100 mM potassium phosphate buffer of desired pH (2.75/3.0) was used as the buffering agent. The reaction was commenced by addition of a mixture of H_2O_2 (2mM) and KCl (1-20 mM) or CPO (2-10 nM). The chlorination substrates were used at the following concentrations: [thionin] = 40 μM and [thiourea] = 100 μM. Control reactions lacked the redox additives, while test reactions were performed by taking additives (amitrol, azide and cyanide) at concentrations ranging from mM to pM. The bleaching of thionin (6.0 × $10^4 M^{-1} cm^{-1}$ at 598 nm) and conversion of thiourea (11.048 × $10^3 M^{-1} cm^{-1}$ at 236 nm) was studied, as the rate is practically zero order for extended initial time periods of assay (for <1.0 min) [26, 27].

Superoxide mediated ATP synthesis and inhibition by CN: KO_2 was purchased from Sigma Aldrich, Na-ATP and NaCN was from SRL Chemicals, Na-ADP and DMSO
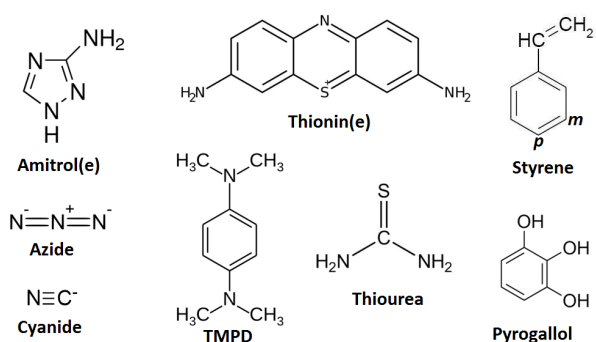
Scheme 4: Structure of ligands and (pseudo)substrates employed in the current study. (structures are not drawn to scale; TMPD- tetramethylphenylenediamine).

Table 1: Sigma values used for the determination of linear free energy (LFE) relationship in chlorination of substituted styrenes, experimentally determined wavelengths of maximal absorption and molar extinction coefficients.

No.	Substitution	Sigma (σ)	λ_{max} (nm)	ε (x 10^4)
1	*p*-OH	-0.37	257	1.31
2	*p*-OCH$_3$	-0.27	257	2.00
3	*m*-CH$_3$	-0.07	249	1.54
4	H	0	247	1.68
5	*p*-Cl	0.227	253	1.91
6	*m*-Cl	0.373	248	1.36
7	*m*-Br	0.391	249	1.34
8	*p*-COOH	0.406	269	2.18
9	*m*-CF$_3$	0.43	246	1.39
10	*p*-CF$_3$	0.54	250	1.57

was from HiMedia, India. A fresh sample of 20 mg KO_2 was dispensed into 1 ml of anhydrous DMSO just before the experiment was commenced and this solution was used as the superoxide stock. All reagents/stocks were filtered/ centrifuged to remove particulate matter. Reactions of a total of 1 ml volume were carried out as per the protocol reported earlier by our group [17]. Experiments were conducted in 2 ml amber centrifuge tubes in Tris-Cl buffer at pH 7.8 (100 mM), with 5 minutes of incubation at 27 °C, after the addition of 10 μl of superoxide stock (or pure DMSO in the respective control) to the made-up aqueous milieu. From the reaction incubate, 10 μl sample was then taken by an auto-injector for analysis by HPLC. Reversed phase HPLC was carried out with the protocol reported by Liu et al. as the guiding principle [41]. A Shimadzu HPLC

Table 2: Program employed for HPLC pumps for separating ADP and ATP

Step	Time (min.)	Solvent mixture
1	Start 0.01 to 2.0	Buffer 100%, CH$_3$CN 0%
2	2.01 to 4.0	Buffer 95%, CH$_3$CN 5%
3	4.01 to 5.3	Buffer 80%, CH$_3$CN 20%
4	5.31 to 15.0	Buffer 75%, CH$_3$CN 25%
5	15.01 to 20.0 Stop	Buffer 100%, CH$_3$CN 0%

system was employed at a constant flow rate of 1.2 ml/min (~1800 psi), with a temporally programmed K-phosphate buffer (pH 7.0, 100 mM) and acetonitrile mixure, as given in Table 2 below.

The reaction product was separated from the reactants using a Shim-pack GWS-C$_{18}$ column and the chromatographic elute was analyzed online with an SPD-M30A Photodiode Array Detector at 254/220 nm for the nucleotide base signal. Internal standards were not used in the reaction mixture and there was no stopping or extraction phase used, in order to minimize cross reactions. Data reported is the average of duplicates.

Analyses, Results & Discussion

Correlation of the lethal dosage of CN with total hemeFe binding sites and other physiological aspects of CN binding: *Recently, we presented a comprehensive calculation and discussion on the total hemeFe binding sites available in blood hemoglobin (Hb) and COX, and compared it to the lethal dose of CN [42] and the following is a brief summation of the same. The human blood has ~2.7 x 10^{22} potential CN binding sites [43, 44]* whereas the lethal blood concentration is ~3.5 x 10^{20} cyanide ions [45]. Therefore, the CN lethal load is only a small fraction of Hb binding sites in blood. A similar assessment shows that there are 10^{21} to 10^{22} binding sites for COX alone [46-48]. In such scenarios, the lethal CN dose is only 1 to 10% of the total vital heme proteins and the analysis does not consider the life-saving dynamic roles of 'relatively non-vital' heme proteins (which could competitively bind to CN, minimizing the latter's access to a more vital heme center like that of COX). An antidote administered for CN toxicity, 4-dimethyl aminophenol, relies on such competitive binding for the elimination of CN in clinical cases [49]. The calculations made above consider CN-hemeFe binding as efficient/irreversible and does not account for losses through non-absorption/non-availability of

the administered CN. However, the ligation of cyanide to hemeFe results via the carbon atom of the CN$^-$ anion and this would be inefficient at physiological pH because the pK$_a$ of HCN is 9.4 [50]. CN is lethal at ~mg/Kg or ~mg/L or ≤ 10^{-6} M concentration levels [51, 52]. Conservatively, >10^2 of the dissociation constant (K$_d$) of the ligand is required to populate >90% of the total hemeFe binding sites. The K$_d$ of CN for COX is comparable to that of oxygen for the reduced enzyme; and CN has very low interaction rate with oxidized COX [53]. In physiological conditions, oxygen is present at >10^{-4} M in the aqueous milieu and its solubility is higher in the lipid phase [54]. As a consequence, the symmetric and smaller O$_2$ should out-compete the asymmetric HCN/CN$^-$ for binding COX. Therefore, physiological explanation for CN's toxicity cannot be a binding-based inhibition of COX function, as the CN binding under these conditions would be inefficient. Further, we have shown that the K$_d$ value determined in vitro with high ligand and enzyme concentrations/ratios are gross over-estimations, with respect to the physiological inhibition/activity [3, 9]. Also, binding of CN (an asymmetric ionic species) to COX (a membrane-bound protein with deep-seated active site) would be even more inefficient, due to issues with availability, partitioning and diffusion constraints. This means that in realistic physiological scenarios, g/Kg or g/L CN concentration (>10 mM) would be needed for effectively inhibiting COX by hemeFe binding, to produce lethal effects. Further, not all of the CN presented is assimilated into the body and a significant amount is also lost to metabolism and excretion. Furthermore, a small amount of injected cyanide should only give a local reaction, but the animal dies quickly and this outcome is seen when CN is administered via inhalation, oral, contact, injection, etc. modalities to diverse life forms. CN inhibits certain heme enzymes' activities even in simple in vitro setups at micromolar IC$_{50}$ levels, which cannot be explained by the higher K$_d$ values of the protein-ligand binding interaction [3, 4, 9].

The functional 20 nM K$_d$ of CN-substate of COX interaction calculated on the basis of activity of the enzyme [55] needs to be re-evaluated under the light of our works. We have shown that electron transfers in such systems occur via diffusible species and not through protein-protein complexations [11]. The low K$_d$ in that system is indicative of DROS-CN interactions. We support our arguments on the following grounds. COX's functional K$_M$ for oxygen is < 0.1 μM [56], which is several orders lower than the experimental K$_d$ of ~0.3 mM [57]. This is theoretically not permissible for the COX-O$_2$ association/reaction. For, the equilibrium/rate constants are positive values, and as K$_M$ = K$_d$ + k_2/k_1, K$_M$ can only be equal to

Table 3: A compilation of binding equilibrium, inhibition and the forward binding rate constants for COX's binding of various Type II ligands at physiological conditions.

Constants	O_2	CO	HCN	H_2S	HN_3
k_{on} [$M^{-1}s^{-1}$]	1×10^8d,e	8×10^4e,m	1×10^2c,e,m	2×10^4a	1×10^6m
K_d [M]	3×10^{-4}d	4×10^{-7}f,m	6×10^{-4}c,m	4×10^{-5}a	6×10^{-5}j
K_M or K_i [M]	$<1 \times 10^{-7}$b	3×10^{-7}l	2×10^{-7}l	2×10^{-7}l	3×10^{-5}l
IC_{50} [M]	-	4×10^{-5}i	5×10^{-5}h	5×10^{-5}g	7×10^{-7}k

The compilation in our publication [42] is expanded here, by including the values for azide.

Key- a- [40]; b- [56]; c- [53]; d- [57]; e- [116]; f- [117]; g- [118]; h- [86]; i- [85]; j- [119]; k- [120]; l- [59]; m- [121].

or greater than K_d. Therefore, the interaction of DROS is imperative in COX reactions to explain for the lower functional K_M. Using murburn concept (DROS-mediation), we had reasoned out similar findings in heme (halo) peroxidases and mixed oxidase (cytochrome P450, CYP) systems [4, 5].

Comparative assessment of hemeFe binding of CN and other small ligands: The shortcomings of binding-based explanation are further highlighted by COX's equilibrium and kinetic constants of CN binding compared with other small heme ligands. Table 3 shows direct experimental K_d, K_M/K_i, IC_{50} and k_1 values from various credible research reports/reviews.

It is known that ferro-porphyrin hemes demonstrate similar binding profiles for diatomic ligands (like CO and CN) that induce a strong field splitting of the Fe atom's *d*-orbitals. Both CN and CO are sigma-donors and pi-acceptors. The rates and the mode of ligand binding also depend on the molecular landscapes- steric hindrances in the active site and on the molecular orbitals' overlaps. CO is considered a stronger ligand of ferrohemes than oxygen or CN (the scientific consensus is that the Fe-CN ligation is weaker than Fe-CO binding), and this enables CO to replace CN in several ferro-porphyrin complexes [58]. H_2S has a pK_a of 7.0 [50]. Therefore, significant amounts of SH^- ions are available at physiological pH, enabling it to be a more effective binder. From literature, it can be seen that kinetic studies mark out CO as a competitive ligand whereas HCN and H_2S are non-competitive inhibitors of COX [40]. Azide is cited as both uncompetitive [59] and non-competitive [60] inhibitor. From Table 3, it is evident that only CO has a markedly lower K_d for COX. It is interesting to note that CO gives an IC_{50} of 4×10^{-5} M, two orders of magnitude higher than the K_d of CO; whereas the non-competitive HCN shows a reverse profile where its IC_{50} value is lower than K_d. This outcome does not agree with the theory of binding based analysis, as shown in Scheme

2. As per Table 3, H_2S should be a more effective inhibitor than CN or CO but this is not the physiological case. It is also interesting to note that CN gives similar IC_{50} and K_i as that of H_2S and CO. Also, K_i of HCN and H_2S for COX is lower than their K_d, which compromises the premises for a binding-based activity assessment. Then, why should CN be more toxic? Clearly, the binding-based explanations draw a blank and cannot effectively reason out an answer. Even while considering the k_{on} rates independently, we cannot explain why CN is more toxic than H_2S or CO. About 50 % CO-Hb is needed for the loss of consciousness and an acute level of >80 % CO-Hb is considered lethal [61]. Even in such overt CO exposures, hyperventilation of the intoxicated patients can help recovery of patients, which is not possible with CN toxicity. Thus, a comparison of the thermodynamic and kinetic binding constants of three toxic gases reveals the inapplicability of hemeFe-ligand binding-based explanations for accounting the toxicities.

Other comprehensive purviews of hemeFe-Type II ligand binding: Wharton & Gibson [62], found the on-rate for O_2 (5.7×10^4 M^{-1} s^{-1}) to be thrice the rate for CO while studying the interaction of *Pseudomonas* COX heme with oxygen and CO. This was when the off-rate for O_2 was four-fold lower than that of CO (i.e. the overall mixture of on-off kinetics were in similar ranges). This could provide support for the argument that big differences in heme enzyme binding cannot be possible if the active site of the protein is not endowed with extremely specific ligand selection capabilities. (In this context, it makes little sense to imagine that COX was evolutionarily screened for better binding with CN.) The observations make realistic sense if we imagined that the COX evolved for fast binding and release of O_2/ROS. We had also demonstrated that toxic heme ligands like N-heterocyclics, azide and cyanide could act as pseudo-substrates at low concentrations. The in situ or in vitro assays show a low functional IC_{50} or pseudo-K_i or pseudo-K_M value of ~10^{-6} M for certain

heme-enzyme reactions, and such observations capture the essence of the physiological toxicity of CN.

Leavesley et al. [63] demonstrated continued cytochrome *c* oxidation and oxygen consumption by COX at even 200 μM levels of cyanide. Also, the presence (or absence) of redox-active additives gives complex temporal profiles, which is not expected with the deterministic binding based theory. If CN binding was efficient, as 200 μM CN is 10^4 fold the purported K_d of COX (as per the value of Jones et al., [55]), the classical explanation dictates that oxygen utilization should cease. But the murburn model is agreeable with the finding that oxygen utilization does not stop (as shown in Scheme 1), and the reduced oxygen uptake could also be owing to its partial recycling by the murburn-CN reactions (to be discussed in detail in the final part of the discussion). These findings conclusively argue against an effective CN-binding based inhibition mechanism, and favor functional 1-electron equilibriums, as proposed by the murburn perspective. Also, cytochrome *c* is reduced even by the 2e donor of H_2S [40], and this fact supports the promiscuous 1e electron equilibriums that form the core of murburn concept. Therefore, from a theoretical study and analyses of available literature on heme-Fe binding, we have shown here that the Fe-CN binding theory is untenable for low dosage regimes. The murburn perspective that "the toxicity difference must primarily be in ROS reaction/interaction dynamics with $CN/CO/H_2S$" is now a worthy alternative for further exploration. This is particularly under the light of the following evidence/arguments supporting the murburn mechanistic theory for oxidative phosphorylation [17, 18]: (1) Starting from ADP and Pi, ROS can form ATP, both in vitro and in situ. (2) Complexes I through IV can make ROS independently and sport ADP binding sites on the matrix side of the apoprotein. (3) ROS titer is directly proportional to NADH/succinate and oxygen presented and ATP produced. Also, ATP cannot be made without the production of ROS. (4) The architecture of mitochondria, structure-function correlation of proteins and cofactors, the multitudes of one-electron redox centers, etc. support the murburn perspective. (5) The ROS mediated synthesis can afford mM level ATP in mitochondria (whereas Complex V mediated equilibrium synthesis can only give picomolar levels of ATP). Under this light, we present our current experimental results.

Probing the mechanism of heme-enzyme mediated moiety transfer (as exemplified in chlorinations by chloroperoxidase) demonstrates the involvement of diffusible radicals: As per the murburn scheme of mOxPhos, ATP formation occurs via one-electron oxidation of the inorganic phosphate ion or via the generation of a one-electron deficient cationic radical (which can recruit the phosphate anion) [17, 18]. This is quite like the chlorination reaction of CPO. Also, in both reaction milieus, the ionic species (phosphate/chloride) must be present at millimolar concentration levels for effective outcomes, as it is difficult to oxidize both these species. When benzylic double bond is subjected to CPO's chlorinating mixture or to other simple inorganic chlorination agents, a diverse array of products are formed, of which αβ halohydrins or keto halides are predominant ([64-66]. Molecules with conjugated sulphur/nitrogen lone pair electrons serve as very efficient substrates for chloroperoxidase-mediated chlorinations, forming S-Cl or N-Cl bonds first, leading to other stabilized products subsequently [67].

Contradicting the claims of the leading research groups that the haloperoxidase mediated chlorination reaction involves an hemeFe-localized species [68-72], a couple of research groups advocated the diffusible species of HClO as the most probable candidate [64, 65, 73, 74]. One of us had resolved this conflict and demonstrated conclusively that the CPO mediated chlorination involved a diffusible species, and showed that the selectivity/specificity shown by the enzymatic system resided in complex interactive equilibriums in milieu [27]. In the current study, we pursued the HPO chlorination reaction's mechanistic implications further. In heme-enzyme mediated chlorine moiety transfer, we use olefins (substituted styrenes) to probe the effect of electron density on the double bond first and then, substrates with π electron conjugated S/N lone pairs are employed to demarcate the effects of ligand and substrate binding. Figure 1 and Table 4 show the results of linear free energy (LFE) relationship studies for the chlorination of select *meta-* and *para-* substituted styrenes (as listed in Table 1). We report (as shown in Figure 1) that the enzymatic mechanism is mechanistically different from diffusible chlorinating species such as HClO (or Cl_2 or Cl_2O, as seen in Table 4), because the heme enzyme mediated rates are several orders higher and the reaction is practically independent of electron density at the double bond. (The latter observation is quite like what one would expect for a highly electrophilic diffusible radical.) However, in the control chlorinating species mixture, upon adding and increasing the concentration of chloride ions, the rate of the reaction increased and the profile suggests lower dependence on electron density at the double bond (Table 4). A recent report had shown the facile formation of superoxide radical from a mixture of pure hydrogen peroxide and excessive hydroxide ions (neutralized by monovalent sodium ions), in aqueous solutions under ambient conditions [75]. This indicates

that radical species generation was facile in such control "molecule-ion equilibriums", even without the d-π electronic catalytic facet afforded by heme enzyme (which was capable of carrying out the reactions in a similar fashion as that observed in the enzymatic mixture). Such an outcome is expected to be further facilitated in CPO's chlorination mixture, a producer of singlet oxygen ($^1O_2$) [76], which in turn, would facilitate superoxide-hydroxyl radical formation via the oxidase pathway. A radical mechanism is evidenced in CPO's chlorination of a beta-diketone [77], and the mechanism in olefin would not be expected to be different. Another group's LFE study had also shown that while synthetic hemeFe model based reaction profiles differed significantly, a diffusible radical (t-butoxy) gave comparable kinetic isotope effects and profiles for N-demethylation reactions catalyzed by the cytochrome P450 reaction system [78]. This reaction is a classical one-electron mechanistic pathway, and it is also relevant for COX assay with a substrate like TMPD [79] (used later in the current study). Therefore, the current LFE study and comparison with literature clearly point to the involvement of diffusible radical species in moiety transfers within the HPO milieu, and this is quite similar to the murburn reaction model of mOxPhos.

Table 5 shows the effect of various Type II ligands (amitrol, azide and cyanide) on chlorinations catalyzed by chloroperoxidase. Thionin is a relatively large trinuclear heterocyclic molecule that would have low probability to access heme center of chloroperoxidase whereas thioruea is a smaller (pseudo)substrate that approaches the dimensions of the ligands studied, and can access the distal heme pocket. Under the reaction conditions, there was little observable OD change when chloride ions were not incorporated into the CPO reaction mixture, indicating that the outcomes reported are owing to chloride ion-chlorine atom/radical equilibrium (or, chlorine moiety transfers). In this context, literature also documents the presence of azidyl, cyanyl and chlorine atom radicals in heme enzymatic or simple control reactions, proven by spin trapping with ESR spectroscopy [80-82].

In the inhibition studies, while CN showed little inhibition at higher proton concentrations for thionin, a millimolar level IC_{50} value was found for thionin and thiourea at lower proton levels. In stark comparison, azide gave micromolar IC_{50} values for both the substrates. Globally, the overall activity profiles were not suited to the classical theory, as R^2 values did not show adherence to the equation for defined inhibition profiles. The same additive proffered a concentration-dependent activation and inhibition of the enzyme activity towards the very same substrate (as seen for the azide incorporating
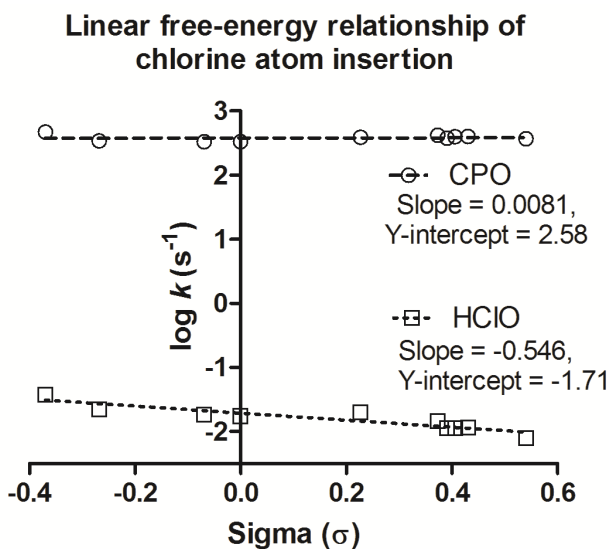

Figure 1: Hammett plots for CPO and hypochlorous acid (HClO) mediated chlorination of substituted styrenes at pH 2.75. The pseudo-first order rates of enzymatic chlorination was more than four orders higher than that of the HClO reaction and the enzyme catalyzed reaction did not show critical dependence on the electron density at the double bond. This was in stark contrast to the inorganic chlorinating agents' reactivity (resulting out of electrophilic substitution). Therefore, the two reactions were mechanistically dissimilar. The reaction mixture contained either 9 nM CPO or 90 μM HClO and 100 μM substituted styrene(s) listed in Table 1.

reactions). This is another feature inexplicable under the classical paradigm, but explicable with murburn concept. CN was a poorer inhibitor of thionin reaction when compared to its interactive outcome seen with thiourea. This result is not expected if this reaction were to be mediated within the distal heme pocket, as thionin is a much larger molecule. Lowering pH by 0.25 units enhanced amitrol's inhibition efficiency for thionin chlorination by two orders of magnitude. This is yet again an observation that cannot be explained by the classical interaction paradigm. The enzyme is only at nM range (and also since the reaction pH/buffering strength is adequate to provide the necessary protons for any active site amino acid residue based interaction with amitrol, whose $pK_a > 4$), the change in inhibition efficiency can only reside in the murburn equilibriums within milieu. In CPO heme-enzyme catalyzed chlorine moiety transfer chemistry, incorporation of HCN shows lesser effects compared to HN_3 (Figure 2 and Table 5), when it is known that the latter is a poorer or comparable ligand (depending on the ambient pH) of CPO's hemeFe. For example- CPO has a K_d of 2×10^{-5} M for HCN and 2×10^{-4} for HN_3 at pH 3 [83]. Therefore, the greater inhibition by

Table 4: A Hammett study of chlorination of p-chloro-, m-bromo-, and p-trifluoromethyl- styrene using various concentrations of chloride ions and with different chlorinating agents (at pH 2.75). Addition of chloride ions and increasing its concentration enhanced reaction rates and alleviated dependency of reaction on electron density at the double bond.

Reactant	Slope (ρ)			Y-intercept (un-substituted rate, s^{-1})		
	No Cl^-	2 mM Cl^-	20 mM Cl^-	No Cl^-	2 mM Cl^-	20 mM Cl^-
H_2O_2	No rxn.	No rxn.	No rxn.	No rxn.	No rxn.	No rxn.
Cl_2	-1.9535	-1.6156	-0.6614	-1.4415	-1.1611	-1.0721
Cl_2O	-1.4757	-1.1404	-0.8598	-1.0687	-0.8174	-0.3323
HClO	-1.2944	-1.1175	-1.0379	-1.1076	-1.0076	-0.5861
$CPO + H_2O_2$	No rxn.	nd	-0.0801	No rxn.	nd	2.609

No rxn.- Within one minute of initiation, the OD of the reaction mixture did not change significantly, when compared to the other mixtures.

azide in CPO's chlorine transfer reaction must be owing to the murburn purview of differences in pK_a (HN$_3$ pK_a = 4.7,) [50], oxidizability of azide and stability azidyl radical. A markedly identical experimental result is seen in the COX reaction [84], where azide showed a significantly lower IC_{50}, compared to that of CN. From Table 3, it can be seen that the K_d of COX for CN is higher than that of azide. This is because at physiological pH, the predominant species is azide anion whereas CN is protonated. This means that for several hemes, though the 'kinetic' k_{on} for azide might be higher in physiological milieu, the stronger 'thermodynamic' Fe-CN ligation makes the two ligands comparable. Once again, it is also inexplicable that the IC_{50} value for COX-N$_3^-$ for chlorination is almost two orders lower than the K_d value. Quite simply, the binding based explanations fail to explain the reaction outcomes.

We had shown that altering the pH or chloride ion strength of the milieu markedly changes the selectivity of the CPO reaction system, both in the enzyme mediated reaction and for the control (hypochlorite) [27]. Therefore, such selective activations/inhibitions seen at dilute concentrations of additives are explicable with the murburn purview. Very importantly, the observations obtained for activation of CPO activity at sub-micromolar or nanomolar levels of CN finds a parallel in COX activities, as reported by some pioneer researchers [85] and in a recent commercial pamphlet of an established reagent provider [86]. In the Wainio and Greenlees paper [85] cited above, their Figures 1 and 2 show this 'activation effect' of low concentrations of CN and Figure 2 shows a similar effect of low levels of CO on COX activity. Similarly, on page 10 of Cayman Chemicals Mitocheck® pamphlet, one can see that there is ~10% activation of COX activity at ~10 nM CN. Such outcomes are not an artefacts; any diligent scientist can cross-check these results. Therefore,

the unusual pH, ion and concentration dependent activations/inhibition profiles indicate the existence of subtle one-electron redox equilibriums in heme-enzyme (and mOxPhos) milieu, that affect the outcomes of moiety transfers.

Probing the mechanism of electron transfer in heme-enzyme inhibition profiles indicate the crucial affects/effects of proton-availability on CN-ROS dynamics: The heme enzyme mediated reactions can lead to fruitful redox metabolism (conversion of the desired substrate to expected product) or wasteful expense of redox equivalents (formation of water and heat). Schemes 1 & 3 show that the heme enzyme cycle can generate ROS like superoxide and hydroxyl radicals and their presence would naturally lead to the formation of stable products like water and/or peroxide within milieu. We investigated the outcome of various CN and pH levels in the heme-enzyme reactions, particularly for the one-electron oxidation of the phenolic compound, pyrogallol. Both HRP and catalase are known to show significant oxidase activity. In the murburn reaction scheme for the model system, pyrogallol's –OH group would be attacked by the DROS as the first step (finally leading to purpurogallin formation). This is quite analogous to the mitochondrial OxPhos where DROS generated by the membrane proteins attack ADP's/Pi's –OH group (finally leading to the esterified product of ATP). Both these reactions also form water. Therefore, this catalase/peroxidase–pyrogallol–peroxide reaction system can be taken as a simple physiological model for the electron transfers of mOxPhos reaction. Since TMPD is a classical assay substrate for COX, the electron abstraction from this substrate (leading to N-dealkylation) is also probed. These models obviate the need for membrane embedded complex systems such as COX, whose erstwhile attributed

Table 5: Effect of various Type II ligands (including CN) and pH/ion concentration on the chlorination of thionin and thiorea by chloroperoxidase. The (purported active site ligand) additives showed highly concentration dependent inhibition and at times, inexplicable activation effects. The non-linear regression analyses showed many times poor fits (with much lower values for R^2, if sub-nanomolar values were also taken for IC_{50} calculation), implying the global inapplicability of classical theoretical foundations.

Substrate, H^+/Cl^-	Cl^-, H^+	Amitrol(e)		Azide		Cyanide	
		IC_{50}	R^2	IC_{50}	R^2	IC_{50}	R^2
Thionin (0.1 mM) Cl^-, 20 mM	H^+, 1.78 mM	2.95 mM	0.95	2.7 µM	0.93	No inhibition	
		Activity recovered below µM ranges		*~90% activity at µM to pM ranges*		*Slightly higher activity than control*	
	H^+, 1 mM	320 mM*	0.4	1.1 µM	0.81	6.5 mM	0.92
		Low activity at µM ranges		*Higher activity below µM ranges*		*No inhibition below µM ranges*	
Thiourea (0.1 mM) H^+, 1.0 mM	Cl^-, 1 mM	No classical inhibition		3.7 µM	0.96	3.3 mM	0.82
		Activity ~90% at all ranges		*Higher activity below nM ranges*		*Activity ~90% even at pM ranges*	
	Cl^-, 20 mM	No inhibition		0.17 µM	0.99	1.1 mM	0.90
		Slightly higher activity at 1 nM range		*Activity recovered at ~ nM ranges*		*Activity recovered at ~100 µM*	

* Select/more values included for IC_{50} determination.
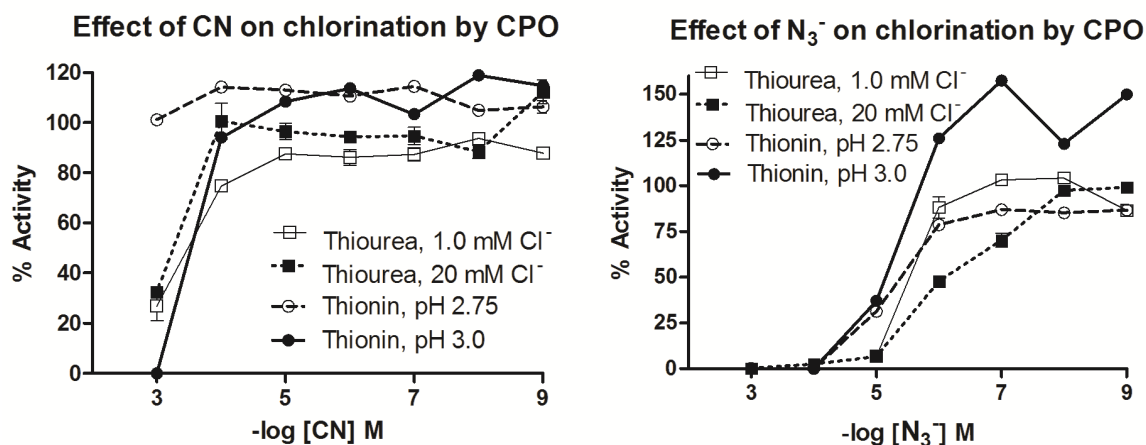

Figure 2: A comparison of activity profiles of CPO mediated bleaching (chlorination) of thionin and thiourea, in the presence of mM to nM levels of CN or N_3^- [4]. At lower concentration of CN, a slight activation can be noted in some of the thionin reaction mixtures. Also, an inexplicably lower level of activity is obtained with sub-micromolar ranges of CN in the thiourea reaction, after activity is recovered at supra-micromolar CN, and then again, activity rises above control value at nM CN. In the azide incorporating reactions, the activations are more pronounced at lower concentrations, in the thionin reaction. Azide, a poorer binder of hemeFe (when compared to CN) gave several orders lower IC_{50} than CN. Such observations cannot be sponsored by binding based outcomes. (For details of experimental setup, please refer methods section.)

function in mOxPhos has been refuted [14]. Also, the earlier purported functionality of "proton pumps + water formation" is practically difficult to trace experimentally.

At pH 5, the pK_a of superoxide (4.8) is approached whereas at pH 9, the milieu is closer to the pK_a of HCN (9.4). Though the physiological pH is 7.4, the functional steady-state regime in the matrix is practically proton-deficient. The mOxPhos steady state scenario is equivalent to a higher pH regime in heme enzyme reactions, which is owing to the "closed reactor" effect brought out by the membranes and the uniqueness of NADH, a "2e + 1H" donor within the mitochondrial reaction. Figure 3 shows the dose response plots of data obtained at pH 9 (which may be compared with the data points/profiles at pH 5

and pH 7, duly provided as non-linear regression traces, within the same figure). At pH 5, the drop from ~80-75% to ~20-25% activity (as indicated from the traditional Hill Slope), the "functionally dynamic" range (where experimental rates are more accurately determined with respect to the controls), is achieved within a single decade of cyanide concentration. In contrast, it takes about three decades of cyanide concentration for the same outcome at pH 9. That is, as the pH increases, the "Hill Slope" flattens. Experimental 'coordination K_d' calculated for nM levels of heme-enzyme to bind small ligands like CN should be >>mM levels [3]. However, the functional K_d value calculated/deduced for CN-heme approaches low µM levels at nM levels of enzyme. For example, in Figure 3, an activity based calculation for the K_d of Catalase-CN interaction is 2.5 µM as follows- 2 nM catalase and 1 µM CN yield ~72% activity of the control (without CN). So, per the Scheme 2, functional K_d at this regime = [free heme] [free CN] / [heme-CN] = (0.72 x 2) (1000 − 0.56) / (0.28 x 2) = 2.5 µM. The highly reproducible data we obtained show that such dynamic K_d values change by orders of magnitude (upon changing the CN concentration) at pH 9, for both catalase and peroxidase (and also for TMPD, not shown). Such "functional K_d" values were smaller at low cyanide concentrations (Figure 3 and Table 6), and this is inconsistent with the theory of binding-based outcomes and the concept of K_d. Such an anomaly with CN can be noted even at pH 7 (result not shown) and this was also noted/deduced from the analyses of data for many heme enzymes and substrates and inhibitors combinations [3, 4, 12]. Since HRP is monomeric, cooperativity is ruled out as the cause for the observed effects. Also, since the allosteric modulation by CN binding at other site(s) on the apoprotein is actually incompatible with the distal hemeFe ligation theory, there is no real theoretically or mechanistically sound explanation in the classical purview. As a consequence, Ockham's razor guides that multiple competing reactions in milieu (the murburn purview) is an appealing option for explaining the observations/results. Figures 3 & 4 show that for various substrates and enzymes, the Hill slope profiles flatten as pH increases. Also, the IC_{50} value falls at pH 7, with higher values at the two pH extremes. This is explained with the fact that the pK_a of the two protagonists (superoxide and cyanide) fall close to these respective pH ranges. Clearly, the outcomes result owing to complex equilibrium catalyses involving protons and diverse species present/generated in milieu, as shown in bottom panel of Scheme 1. Therefore, the murburn model could explain the diverse substrate selectivity/specificity, variable stoichiometries obtained, the various activated enzyme species reported

and complex affects of diverse additive species. Further, the murburn theory can also explain the loss of redox equivalents without high rates of consumption of O_2 (as significant amount of oxygen is regenerated in the futile CN ion-radical cycles). The above study clearly discerns that the high binding affinity (i.e. low 20 nM K_d) that was determined for COX-CN interaction by an activity assay [55] cannot be attributed to "COX's sub-state interactions with CN", but the effect is more due to the practically zero order chemistry of DROS radicals' interaction with CN. This inference is supported by the data from Yonetani & Ray [84]. Table II of their work shows that both CN and ferricytochrome *c* inhibit the COX activity of ferrocytochrome *c* oxidation with an IC_{50} of ~5 x 10^{-6} M. This is possible only if electron transfers in milieu were obligatorily mediated by a promiscuous superoxide, as proposed by murburn concept.

Very importantly, the effect of pH on cyanide inhibition profiles pointed out (and explained) in the model heme enzyme system herein directly correlates to Slater and Chance groups' earlier observations on the existence of multiple pH optima ranges in phosphorylation enzyme inhibition chemistry in situ [87-89]. Quite unlike the usual bell-shaped curves of pH-activity profile for 2-electron chemistry reactions mediated by classical enzymes, the profiles obtained in mitochondrial enzymatic phosphorylation chemistry is similar to the in vitro heme-enzyme mediated 1-electron oxidation of pyrogallol (an −OH group being attacked to give a 2-electron stabilized product of water). These are very strong mechanistic evidences for the influence of milieu proton-equilibriums affecting the outcomes in such 1-electron reactions.

Demonstration of superoxide (DROS) aided ATP synthesis and inhibition of this process by CN: Our earlier studies with heme enzyme CYP and its flavoenzyme reductase in the microsomal xenobiotic metabolic system [5-7, 9, 11, 12] have made it evident that- (i) the flavoenzyme on its own merit gives ROS with NAD(P)H and oxygen and (ii) reactions catalyzed by a particular heme isozyme could be experimentally demonstrated with the heme enzyme in conjunction with superoxide. That is- we have demonstrated that the role of flavin cofactor system is to activate molecular oxygen to give superoxide, which is maintained and used by heme cofactor system in the reaction milieu. Using the indirect activity based chemiluminescence method of ATP detection (with luciferase enzyme), we had recently reported the ROS-assisted in vitro synthesis of ATP (starting from ADP and Pi) [17]. Herein, we traced the in vitro ROS-assisted ATP synthesis with a more direct method (and with elaborate controls), using reverse-phase HPLC. As shown in Figure

Table 6: Determination of functional K_d (extrapolation from experimental inhibition values) for data points of Figure 1 (pH 9) (excluding the ones already mentioned in Figure 1). It is evident that the K_d values calculated from reaction kinetics increase upon increasing the ligand concentration, which is something that does not stand by the basic premises of the binding-based explanation.

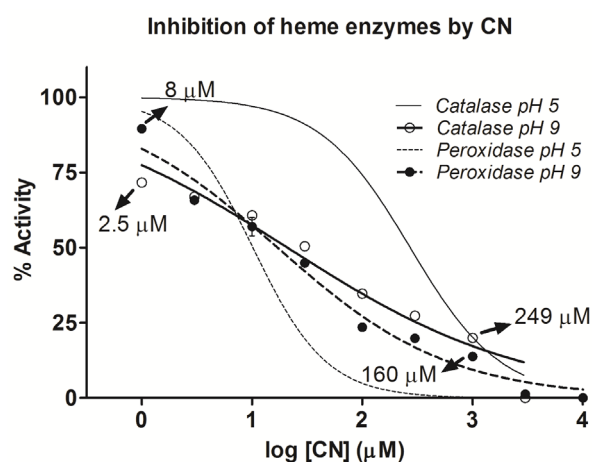| Total CN (µM)	Activity (%)	Free Heme (nM)	Heme-CN (nM)	Free CN (nM)	K_d (µM)
	Catalase (2 nM), pH 9				
300	27	0.55	1.45	299999	*113*
100	35	0.69	1.31	99999	*53*
30	50	1.01	0.99	29999	*31*
10	61	1.22	0.78	9999	*15*
3	67	1.34	0.66	2999	*6*
	Horseradish peroxidase (2 nM), pH 9				
300	20	0.40	1.60	299998	*74*
100	24	0.47	1.53	99998	*31*
30	45	0.90	1.10	29999	*24*
10	57	1.14	0.86	9999	*13*
3	66	1.32	0.68	2999	*5.8*

Figure 3: Data points for CN sponsored inhibition of the formation of purpurogallin (resulting from the one-electron oxidation of pyrogallol) by horseradish peroxidase (HRP) and bovine catalase at pH 9. Functional K_d values estimated at the respective four points (marked with arrows) seem to erroneously indicate that "affinity" increases at low cyanide concentrations. The functional K_d shows a 100 fold and 20 fold lowering when CN concentration is changed by three orders of magnitude, from 1 mM to 1 µM, for catalase and HRP respectively. [HRP or Catalase] = 2 nM; [Pyrogallol or H_2O_2] = 2 mM. Abrupt enhancement of inhibition is seen at CN > 1mM. The non-linear regression plots of the reactions at pH 5 are also provided, to enable a comparison of Hill Slopes.

5 (left panel), the column voided at 2.5 to 3 minutes, whereas the completely separated ATP and ADP eluted at 5.6 and 6.5 minutes respectively. The standard plot of the analytes (middle panel of Figure 5) was made in the range of 0 to 200 µM. The plot gave linear regression R^2 values >0.99; with a practically similar slope of ~6.1 x 10^6 peak area per unit concentration (for 254 nm), for both ADP and ATP. The respective values were used for the estimation of ATP formed and ADP remaining in the reaction mix. A sample chromatogram of a test reaction is shown in the right panel of Figure 5.

The data given in Table 7 shows that the negative controls did not give any ATP at all. With the test reactions, we conclusively establish the viability of ROS-assisted ATP-synthesis in aqueous milieu. The ATP formation levels in the current study agreed with our earlier reported data [17]. Further, in the test reactions, the disappearance of ADP corresponded to the formation of ATP. Very crucially, as seen from Table 7, the incorporation of millimolar levels of cyanide significantly inhibited the in vitro DROS-assisted ATP synthesis. This finding agrees with our earlier reported inhibition of flavoenzyme reductase mediated one-electron reduction of cytochrome *c* [5]. The classical mechanistic perspectives fail to explain how or why CN should inhibit the outcomes in both these reductionist reaction systems but the involvement of diffusible radicals (murburn concept) offers a definite perspective for the same. Earlier reports had pointed out that presentation of radical ROS (like superoxide) could enable ATP synthesis in mitochondria [90] and chloroplasts [91]. In the pioneering experiments of murburn concept [27], it was seen that the addition of a diffusible chlorinating
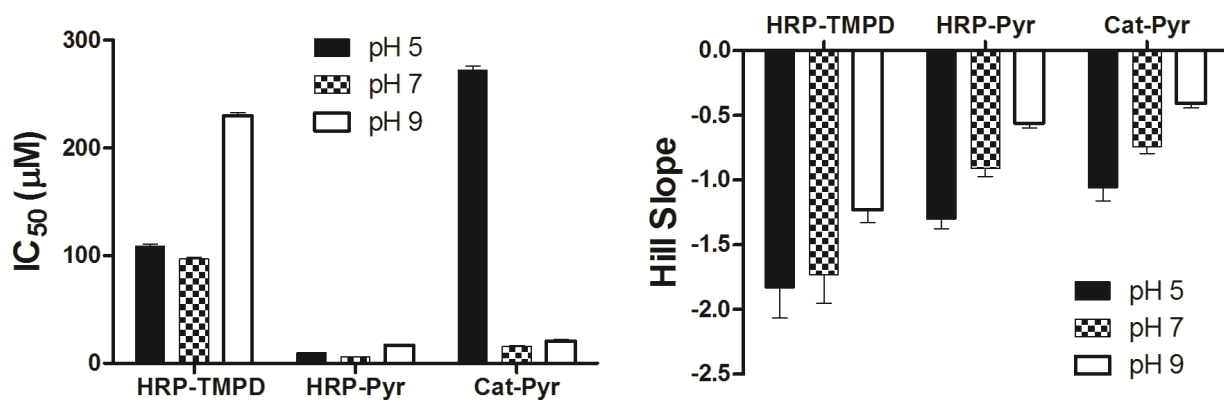
Figure 4: Analysis of earlier reports for the cyanide mediated IC_{50} and Hill slopes for the one-electron oxidation of pyrogallol by HRP and Catalase to give purpurogallin (and dealkylation of TMPD by HRP) at similar environmental conditions, but at varying levels of proton availability [3, 4].

agent like hypochlorite in a single shot at one locus (via a pipette tip) did not adequately capture the high rates or selectivity of the redox enzyme (CPO). However, the selectivity of the enzyme was always the same as that of the diffusible intermediate and the rate/selectivity could be approached by presenting the control chlorinating agent with moderation and with preferred ambience. Likewise, in this study (with mOxPhos chemistry), addition of an unstable DROS like superoxide does not capture the physiological rates and the inhibition with CN was demonstrable only at mM levels. This is because in such free aqueous milieu, DROS can instantaneously react among themselves to produce water. In physiology, the radical DROS are dynamically generated 'discretized' in space and time, in a practically aprotic microenvironment (where water formation is not highly facile due to proton limitation!) and stabilized at discrete loci by the proteins embedded within the inner mitochondrial membrane, thereby enhancing the efficiency of the murburn scheme in such ambience. In actual physiology, as the mitochondrial membrane and proteins ensure a proton limitation cum CN confinement, the inhibitory effect of CN ion-radical would be magnified by many orders. This statement is corroborated by the demonstration herein and elsewhere earlier that inhibition of heme-enzyme/DROS mediated one-electron oxidation of several molecules is critically dependent on proton availability in milieu [16, 9, 4]. We had demonstrated the other effects of ambience (relative protein concentrations and reductant availability) for cytochrome *c* reduction profiles (rates and yields) in a minimalist electron transfer reaction milieu, and inhibitions in such systems were shown even with 100 nM to 100 µM CN [5]. The current report, in conjunction with our earlier reductionist findings, support the physiological relevance of murburn model of ATP synthesis.

A comprehensive stocktaking of our results and comparison with concepts/data from literature: Herein, we employed simple theoretical analyses and reductionist setups to show the mechanistic underpinnings of heme enzymes, with the intent to explicate the complex mOxPhos milieu. We provided here theoretical and experimental evidence to consolidate the negation of classical mechanistic views and support the murburn mechanism of the effect of CN on heme enzyme activity (and thereby, aerobic respiration).

(1) Fungal CPO and inorganic species mediated chlorine moiety transfer was used as a mechanistic model to show the relevance of murburn concept in moiety transfer chemistry. (2) Plant HRP and bovine catalase mediated one-electron oxidation of molecules is used as a probe to demonstrate/understand the complex proton-based redox equilibriums for water formation (the thermodynamic pull, which drives the oxygen-superoxide equilibrium to the right). (3) Incorporation and comparison of some Type II ligands and simple deductions thereof are used to support the murburn explanation of CN toxicity within the context of aerobic respiration. (4) Superoxide mediated ATP synthesis and its inhibition by CN was demonstrated with a more direct (HPLC) assay.

To reiterate and expand upon some points from the introduction- the murburn purview does not negate the classical scheme but incorporates it at high enzyme/substrate concentrations and ratios, and extends beyond the classical purview, into low enzyme concentrations and to regimes wherein enzymes are embedded in phospholipid membranes. As discussed, the interactive equilibrium espoused by the murburn scheme would also have inherent constitutive controls and also explain atypical kinetics, demonstrate diverse substrate preferences (or lack of preferences thereof!)
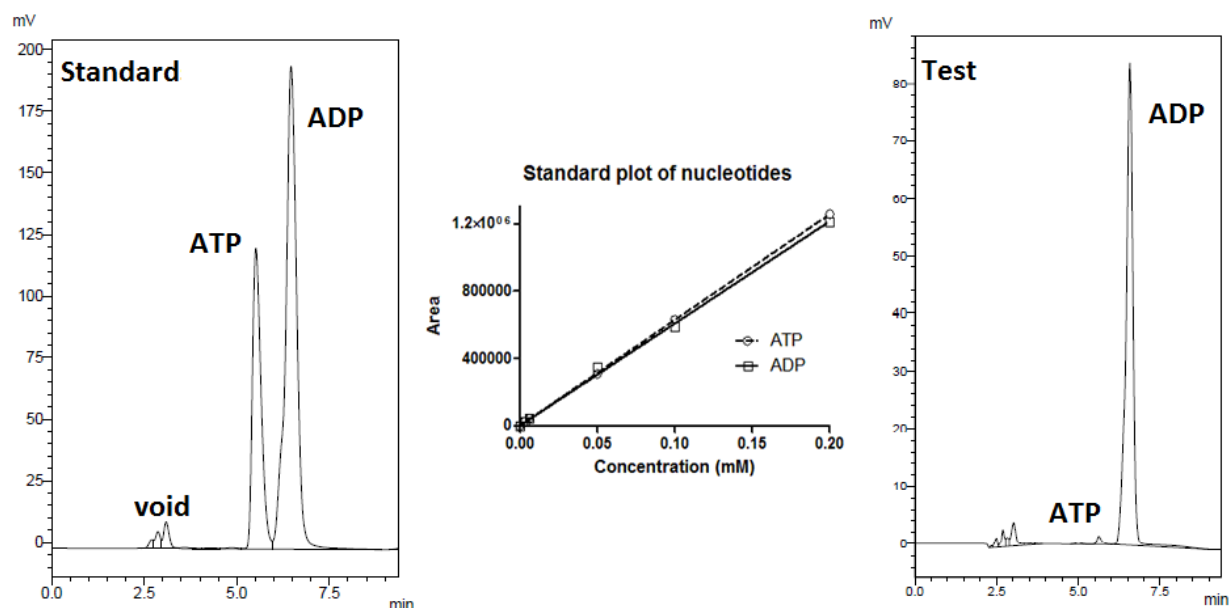
Figure 5: Chromatographic procedures employed for the separation, detection and estimation of ADP and ATP. The left panel shows chromatographic profile of a mixture of high amounts of ATP and ADP (0.33 and 0.76 mM respectively) at 254 nm. The middle panel is the standard plot for ADP & ATP at 254 nm. The right panel shows a sample chromatogram from the experiments (No. 4 of data Table 6). The reaction of ADP+Pi with superoxide showed non-specific products also (eluting after 10 min., result not shown).

Table 7: Estimation of ADP and ATP in reaction milieu by HPLC

No.	Description of sample	Estimated values	
		ADP (µM)	ATP (µM)
1	Method check: Positive control (200 µM ADP + 100 µM ATP)	213*	109*
2	Negative control: 10 mM Pi ± KO$_2$	0	0
3	Negative controls: 200 µM ADP ± (KO$_2$ or 10 mM Pi)	211	0
4	Reaction: Test 1 (200 µM ADP + 10 mM Pi + KO$_2$)	202	2.1
5	Reaction: Test 2 (200 µM ADP + 10 mM Pi + 10 mM CN + KO$_2$)	207	0.7

*A slight overshoot is noted (~6-9 %) in quantitative estimation from the slope value of plot of standards prepared earlier. (This could be owing to evaporation of water from the standards.) Otherwise, estimation showed less than 10% variation in duplicate samples/assays.

with low or lack of enantioselectivity, show diversity in reaction types (hydroxylation, (de)halogenation, phosphorylation, epoxidation, hetero-atom dealkylation, peroxide dismutation, etc.), give variable/non-integral stoichiometry that could lead to disagreements in results across labs (as minute levels of additives could significantly affect the reaction outcomes), explain evolutionary considerations and structure-function correlations of diverse proteins and organelles, account for unusual physiological dose responses, and above all, account for the fast and efficient reactions at low enzyme concentrations. Diffusible radical mediated equilibriums and bimolecular reactions proposed in murburn scheme are more facile and could potentially show much faster rates (~10^{10} M^{-1}s^{-1}). Unlike the classical scheme which deems DROS as an undesired toxic waste product, the murburn scheme sees DROS (particularly, the radical species) as quintessential for electron/group transfers and catalyses. Further, while the classical scheme sees water formation at heme center as the mechanism to explain for redox equivalents' depletion, the murburn model deems stochastic DROS interactions in the milieu as the reason for redox loss/heat generation. Murburn explanation could also rationalize residue mutation studies' outcomes observed in heme-enzyme systems [10,11]. It must be noted that the murburn proposals do not violate any of the fundamental considerations in hemeFe and oxygen species chemistry [92]. Further, the presence and interchanging abilities of various DROS in a dynamic fashion within such reaction systems has been a well-known aspect of redox biochemistry [93-95].

Before analyzing the results of our experiments and values of K_d-IC_{50}, it is opportune to recapitulate in some more detail the earlier relevant/key findings from our group (references duly cited at earlier instances) on diverse redox enzymes belonging to soluble peroxidases and catalases and the membrane-bound mixed oxidase system of cytochrome P450s (CYPs, heme thiolate species) cum reductases (flavin enzyme).

(i) The heme distal pocket concentration plays a higher order role on the formation of a hemeFe-ligand complex. The access to hemeFe is more viable when enzyme is taken at ≥µM level concentrations and at higher ligand:heme ratios. When the enzyme is at low concentration or embedded in membranes and when the ligand is at low concentrations, the access to ionic species and large substrates is challenged by diffusion, solubility and size constraints. For example- (a) Cumene hydroperoxide's access to CPO's hemeFe is limited by size constraints and therefore, this peroxide does not activate the enzyme and form Compound I. However, the very same enzyme can convert this molecule (in its chlorination mixture) and molecules of much larger dimensions, as the latter reaction occurs via a diffusible species. (b) HRP/CPO (10 nM) do not significantly consume 100 µM peroxide by the classical Compound I route. However, with the presence of a heme distal pocket excluded peroxidative substrate, the enzyme milieu can deplete the small hydroperoxide activator. This is by virtue of the enzyme cycling via oxidase pathway and the "thermodynamic pull" exerted by the reactions in milieu, as espoused by murburn concept. That is- redox equivalents are not consumed- or, they are depleted only slowly in the absence of a substrate, but the presence of substrate leads to consumption of redox equivalents. This was earlier erroneously attributed to the heme-pocket binding and Fe-based 'spin redox-switch', as seen in the P450cam model. The new perspective explains outcomes based on substrate's ability to reduce heme-Fe through non-bound modalities and the production of singlet oxygen species by the heme enzymes. Electron pumping through multi-molecular complexes via an outer-sphere model is low on probability scales, and has very low kinetic and thermodynamic viability.

(ii) Ligands like CN, azide and several other molecules/gases and ions (which are oxidizable or reducible additives) can also serve as pseudo-substrates and non-specific redox agents, helping or disrupting electron and group transfers in milieu. This finding has been conclusively supported by Mason's group's findings using ESR spectroscopy, as they have detected radicals from these 'ligands' in several heme enzyme (including COX) milieu [80, 81]. Such works indicate that these molecules serve as (pseudo)substrates, rather than serving as inhibitory active site ligands, in most heme enzyme systems/milieu.

Based on such findings, we have repeatedly pointed out- (a) the radical DROS' ability to serve as electron seekers and carriers in heme enzyme mediated metabolism, (b) heme enzymes serving as DROS stabilizers/modulators and singlet oxygen producers (and not activators of molecular oxygen), (c) several examples of inhibitions and activations sponsored by additives as a result of events occurring beyond the hemeFe center and (d) the role of diffusible reactive species and competing reactions in milieu as the reason for varying stoichiometries and atypical kinetics in these redox enzyme reaction systems. The kinetic data and profiles obtained are therefore, more a reflection of the diffusible species' interactive equilibriums with the enzyme/substrate/additive. It is in this context that the physiological effect of CN and experimental IC_{50} values can be understood and explained.

Although the data points in the IC_{50} study could be plotted with acceptable R^2 values, many heme-enzyme reactions are not actually based in the classical Enzyme-Substrate/Inhibitor binding-based concept [3, 4, 6, 9]. This is evident in several profiles where data points either fall significantly below or rise above the "expected fit", as also noted in the current work. The unusual concentration plots of activity for various Type II ligands attest to our claims. Ockham's razor argues against the supposition that all such diverse enzymes have multiple modalities of binding/interactions with the "evolutionarily unknown, small or large substrates of diverse electrostatic and topological profiles" at the very hemeFe center or distal pocket. Further, increasing the pyrogallol concentration lowered the IC_{50} value of cyanide for the HRP reaction at pH 5, confirming that the effect is NOT due to CN and pyrogallol competitively interacting at the distal heme active site pocket [3]. Furthermore, we have also demonstrated with HRP-TMPD reactions that even with drastically substoichiometric hemeFe : CN ratio of 5 nM : 1.0 to 0.01 nM, significant inhibition is derived [3]. Finally, analyses of in vitro or in silico data obtained from several heme enzymes + additives systems discount heme-active site binding-based explanations. The globally relevant deduction that- multiple competing reactions exist simultaneously, leading to interactive redox equilibriums in the milieu, and protons are a highly crucial reactant/catalyst in such a scheme, affecting both thermodynamics and kinetics of the processes- is more appealing and applicable [15]. Else, we cannot reason observations such as- IC_{50} for CPO-TMPD reaction is practically insensitive to decades of mM CN concentration

as the reaction pH approaches TMPD's pK_a value [3]. Since radicals are stabilized at low concentrations and a radical like superoxide is more stable at low proton levels, the enhanced inhibitory ability at low cyanide concentration at pH 9 is explained. In Figure 3, the abrupt fall in activity levels of catalase/peroxidase from 1 mM to 3 mM CN is owing to the enhancement of murburn scheme by factual heme-binding of cyanide (thereby enhancing the availability of cyanide near the vicinity of heme), making it an even more potent inhibitor. Further, in the classical purview, CN can potentially interact with the hemeFe center via the distal heme pocket prior/post the activation step (thereby, competing with the initial activator or final substrate) and also interact with an allosteric site on the protein (bringing about an influence through non-competitive mode). The latter non-competitive modality and the uncompetitive mechanism (CN binding only to the ES complex) are non-defensible from a theoretical/practical perspective. (The experimental binding is seen at the heme-center, with only the free enzyme! So, the competitive inhibition must result, if the binding based explanation was true.) However, quite like COX, even the model enzymes employed here- HRP and catalase, are known to show a "non-competitive" inhibition outcome with CN [96, 97]. Non-competitive inhibition is one in which the inhibitor is supposed to bind at an alternative site, other than the substrate. In the current scenario, there is little scope to envisage for an "allosteric binding" based conformation or mechanistic change for the bevy of heme enzymes studied. Quite simply, such an evolutionary pressure does not exist. Therefore, the binding-based explanation for heme enzyme-CN inhibition stands falsified by "proof of contradiction" (along the lines employed in the K_M vs. K_d discrepancy obtained from experimental kinetics data for COX) and the "multiple competitive redox reactions in milieu with proton as a crucial limiting reactant" scheme of murburn explanation is deduced as the reason for the outcomes. Also, if DROS served as the catalytic agents and the reactions did not occur at the hemeFe alone (as per the more comprehensive perspective that murburn scheme espouses), then the competitive reactions occurring in milieu (wherein CN is present and could affect outcomes) or heme-pocket would reflect in the experimentally derived kinetic data as "non-competitive" inhibition.

While the mechanism of toxicity of CO is understood to be related to decrease in energy levels and change in redox state owing to metabolic acidosis, the acutely toxic effect of small doses of CN is rather unknown. The research in this area is fraught with ethical issues and want of literature. Only conjectures exist that the CN toxicity results owing to its ability to bind COX. Unlike CO, however, hypoxia is not the reason for the lethal toxic effects of CN [61]. In our recent works with (sub) micromolar levels of heme-enzymes and several toxic small molecules and ions (N-heterocyclics, cyanide/azide, etc.), we had shown/argued that the latter are more likely to act as pseudo-substrates in milieu. This implies that only at higher heme:ligand ratios and high enzyme concentrations, an ion like cyanide serves as an efficient active-site ligand. The in situ or in vitro assays show a low functional IC_{50} or pseudo-K_i or pseudo-K_M (~10^{-6} M) for certain enzyme reactions, and such observations capture the essence of CN's physiological toxicity. We had recently proposed that DROS are ultimately and obligatorily required as catalytic agents for metabolic activities and electron transfer (not just as molecular messengers); particularly for ATP-synthesis and heat generation in mitochondria. Given that the chemiosmotic explanation for cellular respiration is untenable [13, 14, 16-20, 98], the thought lines that "the toxicity difference must primarily lie in DROS reaction/interaction dynamics" makes better rationale, particularly under the light of the structure-function correlations of mitochondrial proteins. The mitochondrial respiratory proteins (Complexes I through IV) show- (i) ADP binding sites, (ii) O_2 accessible redox centers/channels, and (iii) the ability to produce DROS independently. Therefore, the relevant scheme for various protagonists' interaction with COX is projected in Scheme 5. In the physiological schemes (at micromolar levels of cyanide), COX (or other respiratory proteins) are rather unperturbed in their binding/activity for directly interacting with O_2/DROS. (Table 3 shows that k_{on} is the highest for O_2 and it must be remembered that oxygen is available at much higher concentrations and more readily than CN.)

While investigating the mechanism of diffusible radical production from hydroperoxides by heme proteins, Mason's group had found that CN inhibited the production of radical adducts in the milieu [99]. They interpreted the outcome with the classical perspective of cyanide binding to hemeFe. This is unlikely because under identical conditions, if the same heme center can sponsor the production of cyanyl radicals and other radical adducts, then it is the cyanyl radical (and its equilibrium interaction) that inhibits the formation of other radical adducts. If cyanide binding was more facile, then cyanyl radical should not have been produced within the same milieu. The facile formation of cyanyl radical in such systems suggests that CN functions more probably as a (pseudo) substrate, and to a lesser extent as an inhibiting hemeFe ligand. Also, cytochrome c is hexaligated, under physiological scenarios and it is unlikely that CN would

replace the sixth ligation in its coordination sphere. Therefore, we believe that their observations can be taken as direct support of the murburn perspective. More direct evidence favouring our interpretation for the murburn model of mOxPhos was brought out later in Mason's studies on CN inhibition with HRP and COX [100]. They showed that HRP's heme meso carbons were substituted with CN but COX's heme did not show any covalent derivatization in mass spectrometry analysis. They inferred that a protein based thiyl radical formation could be inhibited by cyanyl radical in the COX system. Therefore, three different heme systems were interpreted to be inhibited via three different mechanisms by the same ligand, CN. Once again, we invoke the Ockham's razor against such a deductive rationale for the physiological perspective. (Please see that we do not discount the venerated researchers' data, but we only differ with the interpretation for physiological perspective. The critical insights in heme enzyme functioning we brought out over the last two decades allows a comprehensive interpretation of Mason's group's findings.) The spectroscopic preparations have high absolute concentration of heme proteins and also ligand:hemeFe ratios. We agree that (as the experimental evidence shows) under these regimes, accessing the heme-center or heme edge is probable, and therefore, direct binding to hemeFe or mechanism based inactivation or derivatizaton of heme are probable outcomes. In physiological or routine enzymatic assays or even in the initial time frames of their setups, the condition for heme-based CN inactivation is not met. Also, the heme center of COX is rather occluded, minimizing facile access to triatomic ligands such as HCN. Therefore, COX's inhibition in mOxPhos milieu is predominantly owing to cyanide ion-radical's equilibrium futile cycling within the mitochondria. Reiterating our earlier assertions, the equilibrium and kinetic constants determined for such "murzymes" do not have the same significance as the values interpreted from classical theories of "conventional enzymes" and "substrates" interactions. This is because the kinetic outcome is a reflection of the diffusible intermediates' reaction with the final substrate. It is in this final step where CN comes to play, wherein it interrupts the DROS-aided synthesis of ATP within the matrix. Moreover, if the toxicity of HCN was associated with its metabolism, then we could have a suitable explanation for the dramatic effect of CN on the fundamental powering logic of life. We had proposed the catalytic role shown in Scheme 6 for HCN/CN$^-$ equilibrium in mitochondrial toxicity. As shown, HCN diffuses into the mitochondria, leading to cyanide ion formation, concomitant with DROS
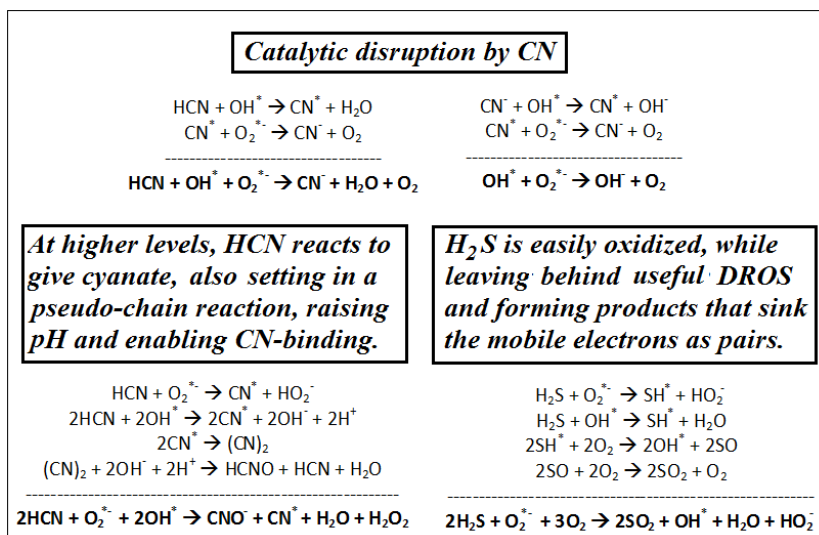
forming water and oxygen. Owing to highly negative charge densities in the mitochondrial membrane (afforded by cardiolipin), CN$^-$ ions get trapped within, and they continue to catalyze futile cycling of superoxide and hydroxyl radicals. Thus, catalytically useful DROS are routed away from oxidative phosphorylation, forming non-productive two-electron sinks. In this scenario, Complex V would deplete ATP reserves for the neutralization of hydroxide or cyanide anions, and cellular energy generation and cellular homeostasis would get disrupted. Later and with higher CN/$^1O_2$ levels, the basic cyanate ion could be formed (thereby increasing pH), enabling cyanide ion ligation to hemeFe, potentially leading to clinical cyanosis. The cellular machinery could further attempt to generate ATP via anoxic modality and this could subsequently lead to acidosis. Our proposals are grounded by the facts that: (A) EPR spectroscopy has been used to demonstrate the presence of CN radicals in heme enzyme systems and cyanate formation has been reported from cyanide [80, 101]. (B) Murburn chemistry toxicity order would be CN > H$_2$S > CO, which is in fact the physiological case [102]. (C) Alternative oxidase can give CN-resistant (photo)respiration, but it is essentially an 'energetically wasteful process'. This process involves a μ-(per)oxo-diiron ligation and protein-localized tyrosine radical [103], and does not invoke hemeFe or diffusible radicals. (D) The lower toxicity of H$_2$S is explained by the new proposal because of the facile oxidation of H$_2$S to SO$_2$ (which could be further oxidized to sulphate, as sulfur moieties are efficient nucleophiles) via a radical process that still leaves catalytically useful DROS in milieu (Scheme 6). (E) Gases HCN and H$_2$S are metabolized in animal and plant systems [104] and this is not possible if heme binding of these gases is effective at low concentrations and if DROS are not the catalytic agents. It is unusual that the non-competitive CN is more lethal than the competitive and effective binder of CO. The oxidase activity of heme enzymes are also inhibited by CN via a non-competitive modality [96, 97]. Since the murburn mechanistic perspective involves ion-radical interactive equilibriums and predicts a non-competitive interaction with CN (that is- not binding at the active site!) in all such systems, the outcomes are explicable. One crucial issue is that the rate constant k_{on} for CN binding with heme proteins is in the order of 10^2 to 10^3 M^{-1} s^{-1} (Table 3). This process can only give rates of $<10^{-3}$ s^{-1} at micromolar concentrations of CN. This implies that the binding-based events need several tens of minutes to hours (depending on the variables involved), to effect in cell/organism death. This slow and reversible binding of CN cannot account for the fast/acute lethality of cyanide, which

[A]

[B]
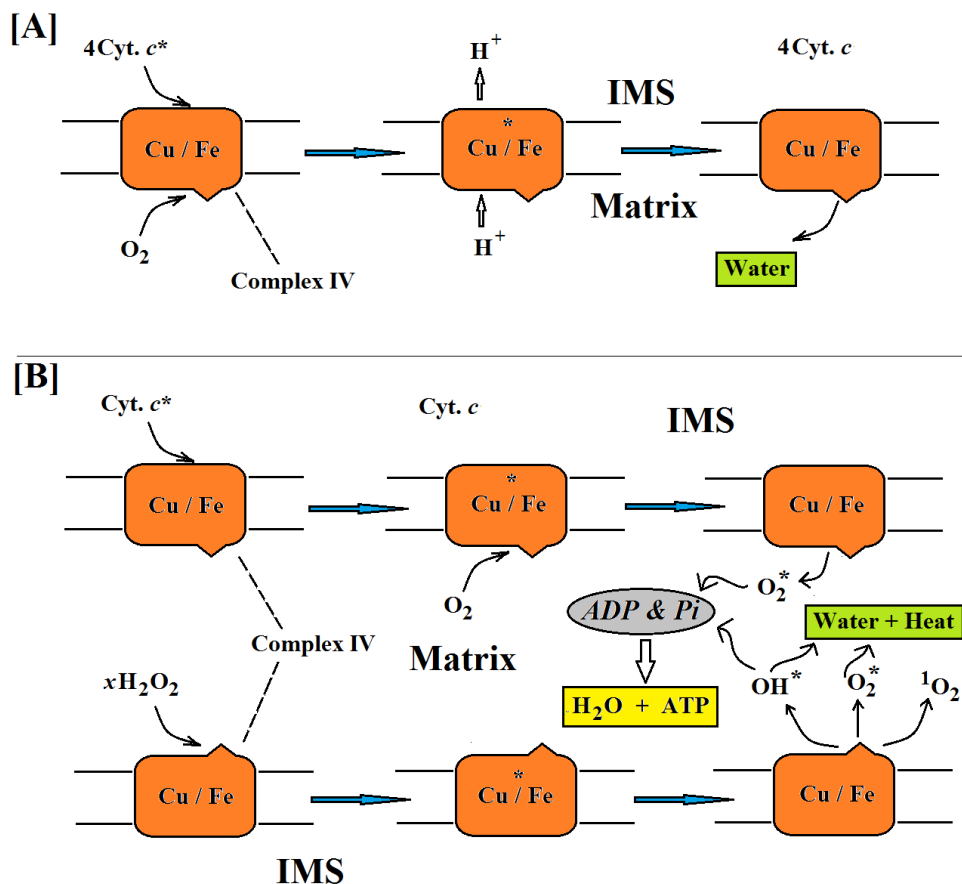
Scheme 5: Complex IV's proposed interaction with cytochrome c, oxygen and peroxide. [A] In the erstwhile mechanism, oxygen merely bound to Complex IV to get reduced by four electrons from four cytochrome c (Cyt. c) molecules and pump out protons on the way to making water at the active site. The hemeFe goes through the two-electron deficient Compound I route. [B] In the new scheme, interaction of both Cyt. c and peroxide is to aid a one-electron scheme within the matrix. The DROS produced attack the ADP (bound on the enzyme) and Pi in milieu to make ATP. The DROS that do not get to react with ADP/Pi react among themselves to make water and heat. A small amount of water formation is possible via Complex IV, but copper may be present to prevent this and to facilitate singlet oxygen formation (which can better activate flavins and get reduced to give superoxide on its own). Details of stoichiometry, electron and proton involvement are not shown. [IMS: inter-membrane space]

occurs in seconds to minutes. Quite contrastingly, ROS (radical) reactions have rate constant k values of 10^8 to 10^{10} $M^{-1} s^{-1}$ (about six orders higher than CN's heme-binding rates) [14], which could give rise to lethal outcomes within second-minutes time scales, explaining the rapid/acute onset of CN toxicity. Also, the stark non-correspondence of K_M and K_d is the result of practically diffusion limited spontaneous DROS reactions at micromolar ranges. This means that the physiological reaction is via diffusible ROS; and oxygen does not stay bound at COX, thereby rendering the ETC concept invalid. Based on our works [3-5], it can be understood that the low K_d value for COX-CN interaction determined by activity assays [55] is a reflection of CN-ROS interactions. These arguments support the murburn perspective that at low concentrations, it is not

via COX hemeFe's direct ligation to the anion that CN proffers physiological toxicity. The catalytic CN ion-radical equilibrium disrupts ROS-mediated ATP synthesis in the vital oxidative phosphorylation routine. This quickly depletes the cellular chemical energy reserves, leading to immediately lethal outcomes. The inhibition of heme enzymes at supra-millimolar levels of cyanide in 'free enzymatic systems (as seen in Figure 3, when the concentration of CN exceeds mM levels, the binding ability increases and therefore, there is a sudden drop in activity) results due to enhanced binding effects. Azide is an efficient bacteriostatic agent at low concentrations because in these prokaryotic systems, oxygen and proton availability is significantly higher (as OxPhos occurs around the cell membrane/periplasm and the periplasm

Catalytic disruption by CN

$$HCN + OH^* \rightarrow CN^* + H_2O$$
$$CN^* + O_2^{*-} \rightarrow CN^- + O_2$$

$$HCN + OH^* + O_2^{*-} \rightarrow CN^- + H_2O + O_2$$

$$CN^- + OH^* \rightarrow CN^* + OH^-$$
$$CN^* + O_2^{*-} \rightarrow CN^- + O_2$$

$$OH^* + O_2^{*-} \rightarrow OH^- + O_2$$

At higher levels, HCN reacts to give cyanate, also setting in a pseudo-chain reaction, raising pH and enabling CN-binding.

H_2S is easily oxidized, while leaving behind useful DROS and forming products that sink the mobile electrons as pairs.

$$HCN + O_2^{*-} \rightarrow CN^* + HO_2^-$$
$$2HCN + 2OH^* \rightarrow 2CN^* + 2OH^- + 2H^+$$
$$2CN^* \rightarrow (CN)_2$$
$$(CN)_2 + 2OH^- + 2H^+ \rightarrow HCNO + HCN + H_2O$$

$$2HCN + O_2^{*-} + 2OH^* \rightarrow CNO^- + CN^* + H_2O + H_2O_2$$

$$H_2S + O_2^{*-} \rightarrow SH^* + HO_2^-$$
$$H_2S + OH^* \rightarrow SH^* + H_2O$$
$$2SH^* + 2O_2 \rightarrow 2OH^- + 2SO$$
$$2SO + 2O_2 \rightarrow 2SO_2 + O_2$$

$$2H_2S + O_2^{*-} + 3O_2 \rightarrow 2SO_2 + OH^* + H_2O + HO_2^-$$

Scheme 6: The disruptive catalysis by CN and comparison of reaction-based inhibitory roles of toxic gases HCN and H_2S in physiological/mitochondrial milieu.

is in equilibrium with the external milieu). In contrast, CN is a more efficient toxic principle in eukaryotic animals (with oxygen and proton-limited systems like the mitochondria). Plants have the alternate oxidase and photorespiration to minimize the effects of CN. Thus, our rationale makes better sense of overall global analyses of CN's effects. It would also be worth exploring if CN's toxicity is altered in systems where Complex V is not obligatorily needed.

Predictability and verifiability of the murburn proposals on CN toxicity: Several CN-based murburn predictive experiments were recently suggested for verifying the proposals and tracing the influence of cyanide in heme enzymes / cellular respiration [18], of which some were reported in the current study. Murburn concept predicts unusual concentration dependent effects. We have shown and also postulated that very small amounts of cyanide may in fact enhance reaction outcomes of heme enzymes, and literature confirms the same result for COX [85]. Such results could be explained by the concentration-dependent rate variability of reactions involved in the molecule-ion-radical equilibrium, as shown in Scheme 6.

Conclusions

The CN-Fe binding-based treatment does not explain the dosage regimes. Also, thermodynamics and kinetics of hemeFe-CN binding-based outcomes do not account for the physiological toxicity of cyanide. Further, the

comparison with ligands like H_2S, CO and N_3^- leads to the inference that a more discretized and irreversible catalytic reaction logic (and not a localized/isolated reversible effect) is the likely causation for the acute toxicity of cyanide. It is now an established idea that unusual in vitro, in situ and physiological dose responses mediated by low concentrations of diverse additives (like redox active small molecules, vitamins, phenolics, N-heterocyclics, azide, etc.) are mediated via the formation of catalytic radicals. Quite analogously, it is proposed that acute lethality of cyanide results due to an ion-radical equilibrium/reaction chemistry. Lethality can be defined as a status where a cell "fails to commission the works needed to sustain life". While oxygen deprivation does not lead to loss of consciousness or lethality for several minutes, administration of cyanide knocks out an animal or cell within seconds. ATP is the known chemical energy currency of the cell, and in the absence of DROS-assisted synthesis of ATP (resulting due to CN mediated catalysis), Complex V's high ATPase activity depletes the cellular reserves of energy. As a result, active powering mechanisms to retain the cellular functions are lost with the presence of cyanide. This inference is strongly supported by CN's ability to inhibit DROS-assisted ATP synthesis, as predicted by murburn purview and amply demonstrated in the current work. Also, the explanation for CN-binding to proteins must be a competitive inhibition process. Going by consensus experimental data reported till date, this is clearly not the case. It is most crucial to note the fact that murburn concept is a "non-competitive"

scheme (not entailing the direct binding of CN to the enzyme active site) that explains the mechanism/kinetics of acute cyanide toxicity. The mechanistic revelations herein directly enable a better understanding of mOxPhos, supporting the murburn model. In the future, the developments from hereon may potentially aid efforts to deal with direct cyanide or natural/synthetic cyanogens' toxicity and also qualify biochemical research (e.g. reason for CN's effect on neuronal action potential) [105-108]. It could also help improve nutrition issues in developing countries, owing to natural cyanogens' implications in dietary intake [109, 110]. On an evolutionary note, the murburn model could also explain why RBCs are anucleated and without mitochondria, and have a lifetime of only a few months. Since these corpuscles have high Fe-porphyrin loads already, the excess hemoproteins from mitochondria would overload the system with DROS, disrupting any possibility for ordered metabolic arrays. Therefore, it was more energetically and evolutionarily viable to eliminate the two organelles of nucleus and mitochondria in cells involved in oxygen transport. Further, it cannot be mere coincidence that cyanide and azide are inhibitors of both the light and dark reactions of photosynthesis [111-113] and in nitrogen fixation too [114]. Clearly, CN and azide are global murburn-active principles, which directly implicates that the mechanistic pathways of these important metabolic routines should be revisited. In this regard, one of us has pointed out several chinks in the currently accepted explanatory paradigm for photosynthesis [115].

Conflict of Interest: Authors state no conflict of interest

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