Biochemical and Biophysical Research Communications 455 (2014) 190–193

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Cyanide does more to inhibit heme enzymes, than merely serving as an active-site ligand

Abhinav Parashar [a], Avanthika Venkatachalam [b], Daniel Andrew Gideon [a], Kelath Murali Manoj [b,*]

[a] Center for Biomedical Research, VIT University, Vellore, Tamil Nadu, 632014 India
[b] REDOx Lab, PSG Institute of Advanced Studies, Avinashi Road, Peelamedu, Coimbatore, Tamil Nadu, 641004, India

ARTICLE INFO

Article history:
Received 17 October 2014
Available online 6 November 2014

Keywords:
Cyanide
Inhibition
Heme-enzymes
Peroxidation
Dose-response

ABSTRACT

The toxicity of cyanide is hitherto attributed to its ability to bind to heme proteins' active site and thereby inhibit their activity. It is shown herein that the long-held interpretation is inadequate to explain several observations in heme-enzyme reaction systems. Generation of cyanide-based diffusible radicals in heme-enzyme reaction milieu could shunt electron transfers (by non-active site processes), and thus be detrimental to the efficiency of oxidative outcomes.

1. Introduction

Anthropogenic activities (from mining and mineral processing industries) present significant amounts of free cyanide in environmental samples, which is measured as the sum total of its ionic and acidic forms (CN^- and HCN, which is henceforth collectively referred to as CN in this text). CN is also present ubiquitously, in trace amounts in plants. It is a serious hazard to all animal life and in the modern era, it is also used as an agent for homicide and suicide. Since CN binds to hemin as well as heme proteins and arrests cellular respiration mediated by cytochrome *c* oxidase (CcO), the mechanism has been studied extensively [1,2].

Inhibition of peroxidase activity of heme-enzymes is explained by the binding of CN as the sixth axial ligand within the distal active site pocket (and this is called Type II binding). A coordination between Fe atom of heme and (most probably) the carbon atom of CN results, thereby inhibiting the formation of the catalytically active species of Compound I [3,4]. Therefore, CN-mediated inhibition of heme-enzyme systems has been followed and interpreted using classical Michaelis–Menten kinetics, and described primarily as competitive and sometimes, as non-competitive [5–7]. Sigmoidal dose response profiles are also frequently seen with CN poisoning [8,9]. Herein, we report several novel observations in heme-CN systems, which challenge long-held notions in the area. It is proposed that inhibition results not just by the virtue of CN binding to the active site, but is also owing to processes occurring in the free solution (sponsored by CN radicals). These findings could provide insight into heme-enzyme function in several *in vitro* conditions and in physiological milieu.

2. Materials and methods

All proteins were purchased from Sigma–Aldrich and other chemicals/reagents were procured from reputed vendors and freshly reconstituted. A systematic study of one-electron oxidations mediated by three structurally and functionally different enzymes-(i) chloroperoxidase (CPO, a kind gift from the late Dr. Lowell P. Hager, UIUC), a heme-thiolate system from the microbial fungus-*Caldariomyces fumago*; (ii) horseradish peroxidase (HRP), a heme-histidylate system from the plant *Armoracia rusticana*; and (iii) mammalian (bovine) catalase (CAT), a heme-tyrosylate system-was carried out. In order to assess the effects of (sodium) cyanide on the above enzymes' activities, three different chromogenic substrates were used-ABTS [2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonicacid)], pyrogallol and TMPD [*N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine].

Abbreviations: ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonate); BMC, Bovine Micrococcus Catalase; CAT, catalase; CcO, cytochrome *c* oxidase; CN, cyanide anion + hydrocyanic acid; CPO, chloroperoxidase; EPR, electron paramagnetic resonance; HCN, hydrocyanic acid; HRP, horseradish peroxidase; LPO, lactoperoxidase; MetMb, metmyoglobin; Pyro, pyrogallol; TMPD, *N*,*N*,*N*′,*N*′-tetramethylphenylenediamine.

* Corresponding author at: Plot #66, 10th Cross Road, Kattur (PO), Trichy, Tamil Nadu 620019, India.

E-mail address: satyamjayatu@yahoo.com (K.M. Manoj).

http://dx.doi.org/10.1016/j.bbrc.2014.10.137
0006-291X/

All reactions were carried out at 27 ± 1 °C in 100 mM potassium phosphate buffer with an initial concentration of enzyme and H_2O_2 as 2 nM and 2 mM respectively. Enzyme or protein concentrations were determined using Soret extinction coefficients of 9.1×10^4 $cm^{-1}\,M^{-1}$ at 450 nM, 1.0×10^5 $cm^{-1}\,M^{-1}$ at 403 nM, 3.2×10^5 $cm^{-1}\,M^{-1}$ at 405 nM and 1.7×10^5 $cm^{-1}\,M^{-1}$ at 409 nM for CPO, HRP, CAT & MetMb, respectively [10,11]. Working stocks of reagents and enzymes were made in distilled water or 100 mM potassium phosphate buffer at pH 6 for CPO & HRP, and pH 7 for catalase & MetMb. Under "steady-state" conditions, the rate of product formation within the initial decades of seconds was used to determine the enzyme activity. Other experimental details, including extinction coefficients of specific products formed are given elsewhere [10,12]. Reactions were carried out in disposable cuvettes comprising 100 µl each of peroxide, substrate and CN (except for control reactions) from 10× stock, 200 µl of buffer from a 5× stock, 20 µl of enzyme from a 50× stock, and finally, distilled water was added to make up the volume to 1 ml. Cascada™ ultrapure biowater (Pall Corp. Ltd., USA) was used at all instances. For MetMb:CN binding studies, 200 µl of buffer from a 1 M stock was made up to a total of 2 ml with distilled water, minus the appropriate volume of protein solution (from 1 mM stock). This was taken in a quartz cuvette and 2 µl of appropriate cyanide solution was sequentially added from different stock solutions to achieve final CN concentrations ranging from 1 nM to 100 µM in the cuvette. GraphPad Prism 5.02 was used to plot and statistically analyze data. Data presented are reproducible, averages of duplicates, with the error bars representing standard deviations. Other details of concentrations employed and other experimental variables are provided in the pertinent legends to the data presented.

3. Results

Select data are shown in Fig. 1 to portray the salient effects of pH change on CN mediated inhibition profiles for various heme-enzyme-substrate combinations. Some profiles show a steep slope, where inhibition is fully effected within a decade of varying CN concentration (as exemplified by HRP-Pyro, pH 8.2 & CPO-TMPD, pH 5). Other profiles show a protracted phase of inhibition that spreads through 2–4 decades of CN concentrations (as seen in Figs. 1 and 2). Though the R^2 values obtained through non-linear fit were >0.9 in all cases (data not shown), there are significant outliers (as exemplified by CPO-Pyro, pH 3 & Catalase-Pyro, pH 5). This indicates violations of the paradigm of the relevant equation employed. Regardless, the IC_{50} values obtained fell within a broad range of $>10^0$ µM (HRP-ABTS, pH 9) to $<10^5$ µM (CPO-TMPD, pH 5). Increasing the pH did not have a unidirectional or predictable effect with respect to a specific enzyme or substrate (or even a given enzyme-substrate combination, data not shown). TMPD reaction with CPO shows a high value of IC_{50} for CN-based inhibition, in the high mM ranges. Since changing pH does not significantly affect CN's binding affinities for these enzymes in the pH range studied [13–15], these observations cannot be explained by CN binding to heme center alone.

The effect of varying substrate concentrations (at a given pH) is presented in Fig. 2. Quite like the non-linear semilog plots in Fig. 1, the linear Hanes–Woolf plot also shows outliers (as exemplified by data in panels D and F). Analysis of the values of IC_{50} or apparent K_M or V_{max} parameters does not vouch for any known inhibition paradigm. For example, the IC_{50} value for HRP-Pyro decreased significantly, when pyrogallol concentration was increased 10-fold (panel B, Fig. 2). This does not agree at all with a conventional competitive inhibition paradigm, where we would only expect the IC_{50} (or apparent K_M) to increase. In these assays, pyrogallol concentration is far below the calculated pseudo-K_M value of ~9.4 mM

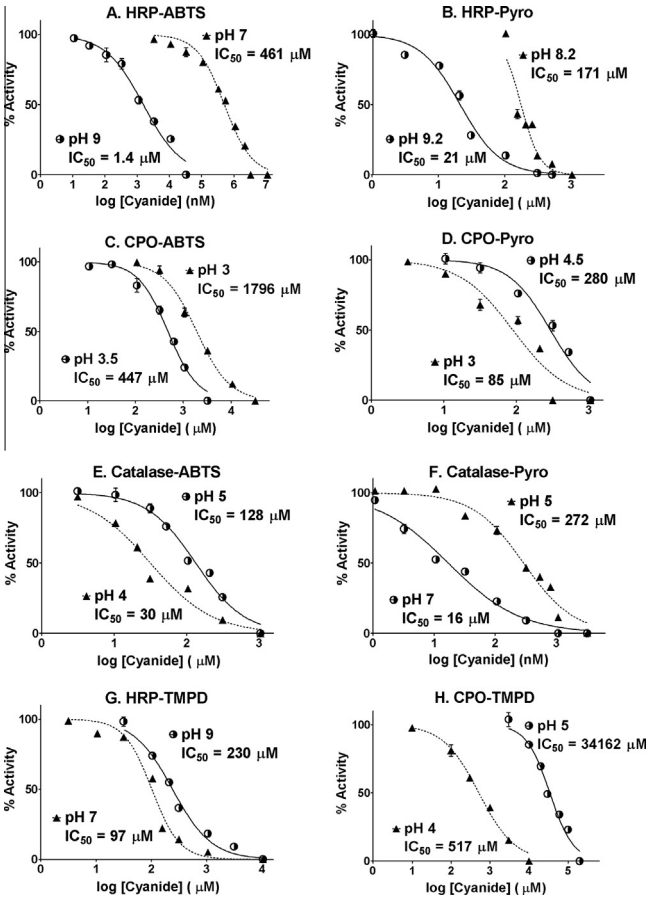

Fig. 1. Effect of pH on dose response (inhibition) profiles for different heme-enzyme-substrate combinations – The non-linear regression fit (for the inhibition data by incorporation of cyanide on different heme-enzyme and substrate combinations) was obtained between the log (inhibitor concentration) vs. normalized response with a variable slope. The respective IC_{50} value is also provided adjacent to the curve, along with the legends and symbols.

(as shown in panel D, Fig. 2). Interestingly, for the same HRP enzyme (at the identical pH of 5), CN seems to show an apparent noncompetitive inhibition for ABTS (IC_{50} and apparent K_M remain constant, when changing inhibitor or substrate concentration). However, higher IC_{50} values and lower apparent K_M were obtained with this substrate (when compared with pyrogallol). The pseudo-K_M was determined to be 0.3 mM for HRP-ABTS (panel C, Fig. 2) under these assay conditions. We know that pK_a of CN is 9.2 and that most ferric heme-proteins have lower K_d to the acidic forms of CN [16]. Therefore, the IC_{50} values obtained in the four profiles for HRP-ABTS (in Figs. 1 and 2) are also inconsistent with the classical non-competitive paradigm.

The highest and lowest IC_{50} values obtained (over a wide range of pH, at comparable concentrations of substrates) for the diverse heme enzymes are given in Table 1. If the rate limiting step was inhibition owing to CN competing with peroxide for the formation of the active enzyme species (as exemplified by Compounds I, II, etc.) alone, then we would not get diverse profiles or significant variations in IC_{50} values for different substrates. If process governing the rates were an active site constrained phenomenon (involving an enzyme active species and the final substrate), then for a given enzyme, we would anticipate the lowest IC_{50} for larger substrate molecules. But that is not the case either. In non-competitive inhibition, since the inhibitor binds to a different site than the substrate, virtually the same inhibitory effect would be expected for an

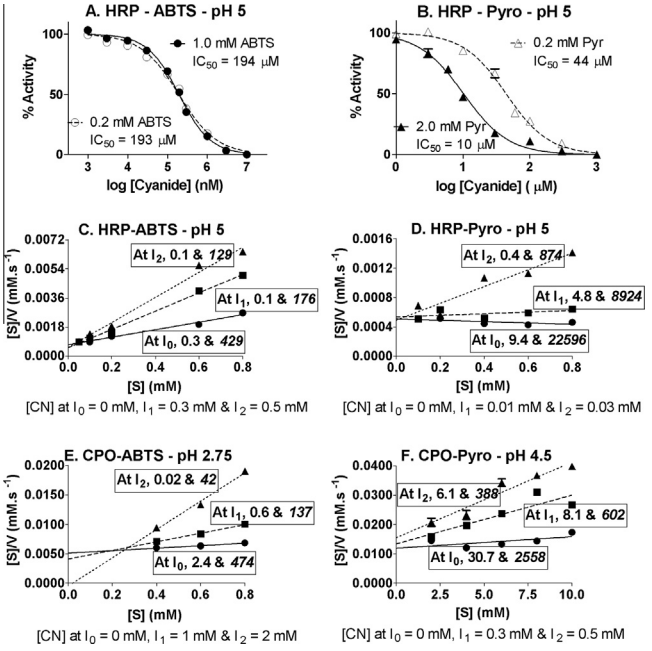

Fig. 2. Effect of substrate concentrations on inhibition parameters – Top Panels A and B show the effect of changing substrate on semilog inhibition dose–response profile curves obtained by inclusion of CN in HRP catalyzed reactions of ABTS and pyrogallol. Panels C–F are the Hanes–Woolf plots for CN proffered inhibition of HRP and CPO reactions, with ABTS and pyrogallol as substrates. Values provided in the boxes refer respectively to K_M in mM and V_{max} (italicized) in s^{-1}.

Table 1
Survey of IC_{50} values for CN in different heme enzyme reaction systems. Both the highest and lowest IC_{50} values are presented in the table (the values obtained for reactions at different pH are separated by a comma). The highest and lowest IC_{50} obtained for HRP-ABTS, HRP-TMPD and HRP-Pyro combinations were respectively-pH (7.0 & 9.0); pH (9.0 & 7.0) and pH (8.2 & 7.0). Similarly for CPO-ABTS, CPO-TMPD and CPO-Pyro reactions, the values were pH (3.0 & 3.5); pH (5.0 & 4.0) and pH (4.5 & 3.0) respectively. And for catalase-ABTS and catalase-Pyro, the respective values were-pH (5.0 & 4.0) and pH (5.0 & 7.0).

Enzyme	High and low IC_{50} (μM) for different substrates		
	ABTS (1 mM)	TMPD (1 mM)	Pyro (2 mM)
HRP	461, 1.4	230, 97	170, 6
CPO	1794, 446	34162, 516	233, 85
Catalase	127, 30	nd	271, 16

enzyme for diverse substrates. Inhibition profiles for the enzyme systems studied do not meet this criterion either.

Further, we explored inhibitions of reactions at very low concentrations of CN, which is shown in Fig. 3. Significant inhibition by CN was observed even at sub-stoichiometric concentrations of cyanide (with respect to the heme enzyme). This clearly points to an inhibition mechanism that is independent of binding of CN binding to the active site or to the protein itself, per se.

4. Discussion

The observations presented challenge hitherto available explanations and invoke upon alternative mechanism(s) for explaining CN-proffered inhibition of the studied enzyme systems-heme-thiolate (CPO), heme-histidylate (HRP) and heme-tyrosylate (catalase) over wide pH/substrate/inhibitor concentration ranges, with substrates belonging to diverse classes-an aromatic amine (TMPD), a phenolic compound (Pyro) and a heteroatomic multinuclear molecule (ABTS).

In hemoprotein research, K_d values are generally determined by taking the proteins at a few μM levels (for the experimental

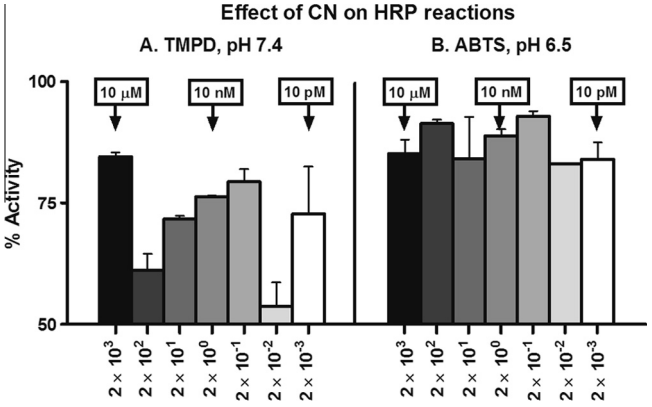

Fig. 3. Effect of cyanide on HRP-mediated peroxidation reactions: Data sets A and B show the effect of ligand (cyanide) pressure on HRP catalysis of two different substrates, TMPD and ABTS at slightly different pH conditions. In (A) CN inhibition of TMPD reactions did not show a normal dose response pattern. In (B) variations in CN concentration did not seem to markedly alter the reaction rates. More importantly, CN still inhibited reactions at sub-stoichiometric concentrations (enzyme: cyanide) in both (A and B). The ligand pressure (enzyme: cyanide ratio) is presented on the *X*-axis. Also, concentrations of CN used ranged from 10 μM to 10 pM (as pointed out by the arrows). The *y*-axis shows % activity w.r.t control (100%). In all reactions, [TMPD]/[ABTS] = 1 mM, $[H_2O_2]$ = 2 mM and [HRP] = 5 nM; 100 mM potassium phosphate buffer was used and temperature was 28 °C.

necessity of spectrophotometric determination by monitoring the hyperchromic lowering/shift of Soret band absorption). The value of K_{CN} for CPO is practically independent of pH from 2.7 to 7, and is equal to ~100 μM [13]. The K_{CN} value for HRP averages ~3 μM in the range of pH 4.2 to 9, and it increases to ~30 μM at ~pH 10 and ~300 μM at ~pH 11 [14]. The K_{CN} value for catalase is reported to be ~10 μM, and this value did not vary within a pH range of 6–8 [15]. In a simple preliminary study, our lab experiments showed that at pH 7 (100 mM phosphate buffer), MetMb has K_{CN} of 14 ± 3 μM and 39 ± 9 μM, when the protein was taken at 5.8 μM and 2.7 μM concentrations respectively. (The absolute values of K_d estimated herein may be slightly higher, due to loss of cyanide during experimentation.) This implies that the binding efficiency is not independent of enzyme concentration, but in fact, is a higher order function of the "active site" concentration. We find confirmation for our observation in Brill and Sandberg's data [17] on Bovine Micrococcus Catalase (BMC). In Figs. 2–4 of their manuscript, data for BMC's cyanide and azide (N_3) complexes are shown. At neutral pH (range of 7.0–7.7), the ligand pressure required to achieve saturation varies as follows-for 7, 70 and 350 μM concentrations of BMC; 920, 82 and 17 folds CN and 3800, 440 and 28 folds N_3 were needed. (The values for cyanide are lower than those for azide because, cyanide is usually a stronger ligand of ferric heme.) Clearly, there exists an inverse relation between the absolute heme-protein concentration taken and type-II ligand concentration required for saturation. It is only a direct inference that K_d would also follow a similar paradigm for all proteins, as confirmed for MetMb in our work. By extrapolation, in the catalytic reaction milieu with nM level heme protein (as employed in our studies), the K_{CN} of the enzymes would be much higher, projected anywhere between 1 and 100 mM. The affinity of cyanide for ferrous heme–iron is very low [18]. Further, CN binding to heme is temporally an on–off equilibrium subjected phenomenon, compared to the directionally driven interaction/reaction of peroxide with heme. Therefore, the percentage of enzyme population (in our assay conditions) that would be bound to CN (at micromolar concentration ranges of CN) at any given instant would be miniscule. This deduction is supported by our findings of incorporating N_3 (a type II ligand of ferric-heme, quite akin to CN but having a negative redox potential) in CPO reactions. It was inferred therein that azidyl radicals enhanced

peroxidations by serving as an efficient "secondary" catalyst [12]. The activation by azide and inhibition by cyanide (when both are type II binders) could perhaps be explained by the large difference in their redox potentials.

Mason's group had confirmed cyanide-based (cyanyl) radical formation in HRP (horseradish peroxidase), CPO (chloroperoxidase), LPO (lactoperoxidase) and MetHb (methemoglobin) systems in the presence of H_2O_2 [19,20] using EPR spectroscopy. We have also observed chaotic activation and inhibition modulations of heme-enzyme reactions by several molecules, taken at low concentrations, which were attributable to diffusible radical generation in the reaction milieu [12,21,22]. Therefore, a highly probable explanation for the gamut of observations reported herein is that cyanide also inhibits heme-enzyme reactions (particularly at low ligand pressures and low enzyme concentrations) through a non-active site role within the reaction milieu. In the last few years, we have invoked upon the complex role of diffusible species and reaction milieu networks to explain for several atypical heme-enzyme phenomena, as exemplified by- substrate inhibition, enhancements/inhibitions of activities by additives and other hitherto active-site or binding-associated phenomena [21–23]. The roles of enzyme's active site amino acid residues in determining the fate of substrate binding and catalysis is well charted. The new ideas on heme enzyme reaction paradigm revealed here calls for a detailed investigation on the attributes of substrates like- redox potential, pKa, log P/D, dimensions, etc. and their roles in determining the competing reactions occurring in milieu. Further, it is opportune to caution that defining heme enzyme reactions with standard terms like IC_{50}, K_i, K_M, etc. may not be technically appropriate.

Acknowledgments

This work is dedicated to the late Lowell P. Hager, Professor Emeritus at UIUC (Member, NAS-USA), for his truly inspiring mentorship. The fundamental strings leading to this work were discovered in the 1990s at his lab at UIUC, when K.M.M. worked with him as a postdoctoral research associate. K.M.M. acknowledges support from DBT, Government of India.

References

[1] W.E. Van Heyningen, The inhibition of respiration by cyanide, Biochem. J. 29 (1935) 2036–2039.
[2] S. Yoshikawa, D. O'Keeffe, W. Caughey, Investigations of cyanide as an infrared probe of hemeprotein ligand binding sites, J. Biol. Chem. 260 (1985) 3518–3528.
[3] M.H. Smit, A.E.G. Cass, Cyanide detection using a substrate-regenerating peroxidase-based biosensor, Anal. Chem. 62 (1990) 2429–2436.
[4] T. Poulos, S. Freer, R. Alden, N.H. Xuong, S. Edwards, R. Hamlin, J. Kraut, Crystallographic determination of the heme orientation and location of the cyanide binding site in yeast cytochrome c peroxidase, J. Biol. Chem. 253 (1978) 3730–3735.
[5] B. Chance, The kinetics of the enzyme-substrate compound of peroxidase, J. Biol. Chem. 151 (1943) 553–577.
[6] B. Chance, The reaction of catalase and cyanide, J. Biol. Chem. 179 (1949) 1299–1309.
[7] M. Sono, J. Dawson, L. Hager, The generation of a hyperporphyrin spectrum upon thiol binding to ferric chloroperoxidase. Further evidence of endogenous thiolate ligation to the ferric enzyme, J. Biol. Chem. 259 (1984) 13209–13216.
[8] C. Piantadosi, A. Sylvia, F. Jöbsis, Cyanide-induced cytochrome a, a3 oxidation-reduction responses in rat brain in vivo, J. Clin. Invest. 72 (1983) 1224–1233.
[9] H.B. Leavesley, L. Li, K. Prabhakaran, J.L. Borowitz, G.E. Isom, Interaction of cyanide and nitric oxide with cytochrome c oxidase: implications for acute cyanide toxicity, Toxicol. Sci. 101 (2008) 101–111.
[10] A. Parashar, K.M. Manoj, Traces of certain drug molecules could enhance heme-enzyme catalytic outcomes, Biochem. Biophys. Res. Commun. 417 (2012) 1041–1045.
[11] E. Antonini, Interrelationship between structure and function in hemoglobin and myoglobin, Physiol. Rev. 45 (1965) 123–170.
[12] D. Andrew, L. Hager, K.M. Manoj, The intriguing enhancement of chloroperoxidase mediated one-electron oxidations by azide, a known active-site ligand, Biochem. Biophys. Res. Commun. 415 (2011) 646–649.
[13] A.-M. Lambeir, H. Brian Dunford, M.A. Pickard, Kinetics of cyanide binding to chloroperoxidase in the presence of nitrate: detection of the influence of a heme-linked acid group by shift in the appa, J. Inorg. Biochem. 19 (1983) 291–300.
[14] W.D. Ellis, H.B. Dunford, The kinetics of cyanide and fluoride binding by ferric horse-radish peroxidase, Biochemistry 7 (1968) 2054–2062.
[15] F. Millar, J.M. Wrigglesworth, P. Nicholls, Ligand binding to catalase and metmyoglobin, Eur. J. Biochem. 117 (1981) 13–17.
[16] J.J. Christensen, R.M. Izatt, D. Eatough, Thermodynamics of metal cyanide coordination. V. Log K, ΔH° and ΔS° values for the Hg^{2+}-CN-system, Inorg. Chem. 4 (1965) 1278–1280.
[17] A.S. Brill, H.E. Sandberg, Spectral studies of iron coordination in hemeprotein complexes: difference spectroscopy below 250 mμ, Biophys. J. 8 (1968) 669–690.
[18] M. Sono, J.H. Dawson, L.P. Hager, Ligand and halide binding properties of chloroperoxidase: peroxidase-type active site heme environment with cytochrome P-450 type endogenous axial ligand and spectroscopic properties, Biochemistry 25 (1986) 347–356.
[19] Y.R. Chen, L.J. Deterding, K.B. Tomer, R.P. Mason, Nature of the inhibition of horseradish peroxidase and mitochondrial cytochrome c oxidase by cyanyl radical, Biochemistry 39 (2000) 4415–4422.
[20] S.N.J. Moreno, K. Stolze, E.G. Janzen, R.P. Mason, Oxidation of cyanide to the cyanyl radical by peroxidase/H_2O_2 systems as determined by spin trapping, Arch. Biochem. Biophys. 265 (1988) 267–271.
[21] A. Parashar, S.K. Gade, M. Potnuru, N. Madhavan, K.M. Manoj, The curious case of benzbromarone: insight into super-inhibition of cytochrome P450, PLoS ONE 9 (2014) e89967.
[22] S.K. Gade, S. Bhattacharya, K.M. Manoj, Redox active molecules cytochrome *c* and vitamin C enhance heme-enzyme peroxidations by serving as non-specific agents for redox relay, Biochem. Biophys. Res. Commun. 419 (2012) 211–214.
[23] K.M. Manoj, A. Baburaj, B. Ephraim, F. Pappachan, P.P. Maviliparambathu, U.K. Vijayan, S.V. Narayanan, K. Periasamy, E.A. George, L.T. Mathew, Explaining the atypical reaction profiles of heme enzymes with a novel mechanistic hypothesis and kinetic treatment, PLoS ONE 5 (2010) e10601.