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RESEARCH ARTICLE

Selective binding of divalent cations toward heme proteins

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Abstract **Potential toxicity of transition metals like Hg, Cu and Cd are well known and their affinity toward proteins is of great concern. This work explores the selective nature of interactions of Cu^{2+}, Hg^{2+} and Cd^{2+} with the heme proteins leghemoglobin, myoglobin and cytochrome C. The binding profiles were analyzed using absorbance spectrum and steady-state fluorescence spectroscopy. Thermodynamic parameters like enthalpy, entropy and free energy changes were derived by isothermal calorimetry and consequent binding parameters were compared for these heme proteins. Free energy (ΔG) values revealed Cu^{2+} binding toward myoglobin and leghemoglobin to be specific and facile in contrast to weak binding for Hg^{2+} or Cd^{2+} . Time correlated single photon counting indicated significant alteration in excited state lifetimes for metal complexed myoglobin and leghemoglobin suggesting bimolecular collisions to be involved. Interestingly, none of these cations showed significant affinity for cytochrome c pointing that, presence of conserved sequences or heme group is not the only criteria for cation binding toward heme proteins, but the microenvironment of the residues or a specific folding pattern may be responsible for these differential conjugation profile. Binding of these cations may modulate the conformation and functions of these biologically important proteins.**

Keywords heme proteins, divalent cations, fluorescence quenching, isothermal calorimetry, time correlated single photon counting (TCSPC)

Introduction

The interaction between proteins and other small molecules or ions is fundamental to all biological functions. The binding of a small molecule or ions (ligand) to a protein is greatly affected by the structure of the protein and is often accompanied by conformational changes and modulation in biological functioning. Each protein has different activity and also they interact in site specific manner. The important aspect is that they are active only in a specific conformation e.g. in a specific tertiary or quaternary structure and a change in this conformation may turn the protein to be non-functional. The changes may include denaturation, pH variation, temperature change or ligand binding. In this work, interaction of three metal cations (Cu^{2+}, Hg^{2+}, Cd^{2+}) with heme proteins leghemoglobin (Lb), myoglobin, (Mb) and cytochrome C (Cyt C) has been explored and compared. Heme proteins contain an iron porphyrin as the prosthetic group, and a globin moiety to regulate their functional activities The diversity in functions of theses heme proteins are dictated by several factors like the number and nature of the axial ligands, the spin and oxidation state of the iron and the nature and folding of the polypeptide chain around the active site. Researchers have taken special interest in porphyrins because of their stability as well as their chemical, photophysical, photochemical, electrochemical and catalytic properties in many areas (Kadish et al., 2010) and due to their fundamental importance in proteomics, medical diagnostics, and pathogen detection (Pinto and Schanze, 2004). Leghemoglobin (Lb) is a heme containing monomeric protein, which was isolated from nodules of the roots of the *Arachis hypogea,* the molecular weight being nearly 15–17 kDa. Lb, found in root nodules of legumes, acts as a nitrogen fixer by scavenging free oxygen. It buffers the concentration of free oxygen in the cytoplasm of infected plant cells to ensure the proper function of root nodules (Basak and Bhattacharyya, 2013). Lb has a high affinity for oxygen (a K_m of about 0.01 μM), about ten times higher than the β chain of human hemoglobin. This allows an oxygen concentration that is low enough to allow

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nitrogenase to function but high enough to provide the bacteria with oxygen for respiration. This monomeric Lb binds to and transport molecular oxygen maintaining a low oxygen environment within the root nodule. This permits the micro aerobic bacteria to convert atmospheric dinitrogen into ammonia, which is then assimilated by the plant for growth. Interestingly, Lb and myoglobin (Mb) have almost identical structure and similar non-covalent heme interactions. However, soybean Lb exhibits approximately 20-fold greater affinity for oxygen compared to myoglobin (Pinto and Schanze, 2004), though nothing is known about the structure and function of Lb from *Arachis hypogea*. The amino acid sequence of the globin moiety of Lb depends on the legume species, even within the same species, several isoproteins or Lb components are usually found which differ from each other by a few amino acids. Thus, we selected two well known monomeric heme proteins Mb and Cyt C to compare with Lb by probing through their binding profiles with divalent cations .

Cytochrome C, the essential ancient protein, performs a key step in the production of cellular energy, and it has changed little over millions of years. Cyt C (Mw12.4 kDa) consists of a single polypeptide chain of 104 amino acid residues including two tryptophan residues and a covalently attached heme group (Harbury and Loach, 1960; Dickerson and Timkovich, 1975; Marchon et al., 1982; Wuthrich, 1985; Raphael and Gray, 1991). The heme is surrounded by tightly packed hydrophobic side chains, only one edge of the planar heme ring is accessible to the surface. Small channels on either side of the ring permit only small hydrophobic molecules to the heme iron. The non-polypeptide globular part is covalently bonded with heme and essential for the physiochemical activity of Cyt C. Myoglobin is also a monomeric heme-protein of molecular weight 17.7 kDa. It is a single-chain globular protein of 154 amino acids including two tryptophan residues (Ordway and Garry, 2004). It contains a heme prosthetic group non covalently attached at the center around which the remaining apoprotein folds into eight alpha helices. It is the primary oxygen-carrying pigment of muscle tissues (Harbury and Loach, 1960). This protein acts as oxygen transporter in our body enabling the directed movement of oxygen into, out of or within a cell, or between cells. It is involved in the transport of a molecule (metabolite, protein, etc.), an ion or an electron across cell membranes, inside the cell or in a tissue fluid.

Divalent metal ions are essential for life as trace elements but after exceeding a certain concentrations they become toxic. Heavy metals are difficult to remove from the environment causing a global environmental hazard and unlike many other pollutants, they cannot be chemically or biologically degraded. Agricultural soils are mainly contaminated with Cd^{2+} due to the excessive use of phosphate fertilizers, dispersal of sewage sludge and atmospheric deposition. Thus, contamination by Cd^{2+} is increasing both in human food and in overall agricultural environment and there is enough chance that plant proteins like Lb can be exposed to Cd^{2+} in soil. High concentrations of copper produces oxidative damage to biological systems, including peroxidation of lipids or other macromolecules. While the cause and progression of Alzheimer's disease are not well understood, research indicates that, among several other key observations, copper accumulate in the brains of Alzheimer's patients, though it is not yet confirmed whether this accumulation is a cause or a consequence of the disease. Mercury toxicity has been referred to as the "invisible epidemic," since the impact is not immediately noticed and builds over time. Mercury is cumulative poisonous metal whose compounds found in the environment mainly originated from industrial sources (Mata et al., 1997). Mercury showed high affinity for sulfhydryl groups present in proteins (Murayama, 1958), if they are not available other groups efficiently compete for the metal.

The interactions of proteins with ligands have received a great deal of interest for many years due to their application in a great variety of industrial, biological, and cosmetic system (Takeda and Hachiya, 2002). In this work we have explored the binding profile of three cations Cu^{2+}, Hg^{2+} and Cd^{2+} with the monomeric heme proteins leghemoglobin, myoglobin and cytochrome c utilizing calorimetric and spectroscopic techniques. Spectrophotometry and spectrofluorimetry were used to estimate the binding affinity, binding sites, Stern-Volmer quenching constant whereas thermodynamic parameters like enthalpy, entropy, free energy were worked out using isothermal calorimetry (ITC) and finally Time correlated single photon counting (TCSPC) was used to explore excited state lifetime of the bound complexes. This investigation provides an estimation regarding selective affinity of cations toward different heme proteins to assess their potential risk for toxicity.

Experimental

Sample preparation

Lb was isolated and purified from root nodules of *Arachis hypogea* (Basak and Bhattacharyya, 2013). Mb and Cyt C were purchased from Sigma Company for all the following experiments. Fresh aqueous stock solutions of the proteins were prepared and diluted according to the requirement of the experiments.

UV-Vis absorbance and steady state fluorescence measurements

Absorption measurements were carried out in a Jasco V630 double beam UV–Vis spectrophotometer. All steady-state fluorescence measurements were performed in Varian carry eclipse spectrofluorimeter. Fluorescence quenching measurements were recorded for Lb and other heme proteins Cyt C

and Mb (15 μM) titrated with increasing concentrations of $CuCl_2$, $HgCl_2$ and $CdCl_2$ (0 to 70 μM) at 22°C. These samples were excited at 295 nm, and the fluorescence emission was recorded in the range of 310 to 400 nm. The possible quenching mechanism can be interpreted by analyzing the Stern-Volmer plot and modified Stern-Volmer plot of the proteins and divalent cation interactions. Stern–Volmer equation can be applied in the following form: $F_o/F = 1 + K_{sv}[Q]$, Where F_o and F are the steady-state fluorescence intensities in absence and presence of quencher respectively, $[Q]$ is the concentration of the quencher, and K_{sv} is the Stern Volemer quenching constant, measuring the efficiency of quenching. The Stern –Volmer plot (Fig. 2A) was analyzed to obtain various binding parameters for the interaction of divalent cation. The plot of F_o/F versus $[Q]$ gives a straight line and K_{sv} was worked out from the slope. The Stern volmer equation, R^2 value, $K_{sv} \times 10^6$ mol^{-1}, modified SV equation log $(F_o\text{-}F/F) = \log k_a + n \log [Q]$, (Lehrer, 1971) and ln k_b/ mol^{-1} have been recorded in Table No 1. (Lakowicz, 2006) Modified SV equation describes, the fraction of fluorescence quenched upon full ligation compared to the integrated protein. The binding constant (K_a) and the number of binding sites (n) can be calculated according to the equation: $\log(F_0 - F)/F = \log K_a + n \log[Q]$, (Kang et al., 2004) where F_0 and F are the fluorescence intensity without and with the ligand, respectively (Zhang et al., 2009). A plot of log $[(F_0–F)/F]$ vs. log $[Q]$ gave a straight line (Fig. 2B) whose slope is equal to n (binding sites) and the intercept on Y-axis to log K_a (K_a equal to the binding constant). The linear coefficient R^2 indicated that the assumptions underlying the derivation of Equations were satisfactory. Free energy of the reaction was calculated by using the following equation: $\Delta G = -RT\ln K_a = RT\ln K_d$, (Arias-Moreno et al., 2011), where Δ G is the Gibbs energy of binding, K_a is the association equilibrium constant, and K_d is the dissociation equilibrium constant . Analysis of Stern–Volmer plots in this system yields equilibrium expressions for quenching constants which are analogous to associative binding constants for the quencher–acceptor system (Murphy et al., 2004).

Isothermal titration calorimetric measurements (ITC)

The thermodynamic aspect of binding of ligand with macromolecule is best understood by ITC and is characterized by the stoichiometry (n), the association constant (K_a), the free energy (ΔG), enthalpy (ΔH), entropy (ΔS), and heat capacity of binding (ΔC_p). In combination with structural information, the energetics of binding provides a complete analysis of the interaction and assist in identifying the most important regions of the interface and the energetic contributions. ITC measures the binding equilibrium directly by determining the heat evolved on association of a ligand with its binding partner. In a single experiment, the values of the binding constant (K_a), the stoichiometry (n), and the enthalpy of binding (ΔH) are determined. The free energy and entropy of binding are determined from the association constant. In our study the energetics of binding of divalent metal cations with heme containing proteins Lb, Mb and Cty C were measured by ITC.

The enthalpy was determined using the temperature dependence of the equilibrium constant as expressed in the Van't Hoff equation:

$$(\delta \ln k/\delta T)_p = \Delta H/RT^2$$

where k is the equilibrium constant (or association constant. T is the absolute temperature in Kelvin and R is the gas constant. ΔH can be directly obtained from ITC experiment.

Free energy can be determined directly from the binding constant value using the equation:

$$\Delta G = -RT\ln K$$

If ΔG and ΔH are known then using the equation:

$$\Delta G = \Delta H - T\Delta S, \Delta S \text{ may be determined}$$

where ΔS indicates the entropy change value at temperature T.

The energetics of the binding of $CuCl_2$, $HgCl_2$ and $CdCl_2$ to Lb and other heme proteins like Cyt C and Mb (15 μM) at 22°C were measured using a VP-ITC titration microcalorimeter (MicroCal Inc., Northampton, MA). The sample and reference cell of the calorimeter were loaded with Lb, Mb, and Cyt C solution each of 300 μL (3 μM) and 100 mM sodium phosphate buffer (pH 7.0). The protein and ligand concentration were fixed at 16 μM and 9 mM respectively. Then multiple injections of 80 μL of $CuCl_2$, $HgCl_2$ and $CdCl_2$ solution (60 μM) were made into the sample cell containing Lb, Mb, and Cyt C consecutively till saturation was reached for each protein separately . The plot of the heat evolved (kcal) per mole of Lb added, corrected for the heat of Lb dilution, against the molar ratio of Lb to different cations incubated for 5 d and data (filled squares) were fitted to a sequential binding sites model, and the solid lines represent the best fit. The basic method of ITC implies ligand concentration be kept 30-40 times more than protein concentration and a significant concentration to be maintained for the protein (Zaidi et al., 2013; Basak et al., 2015). Heats of dilution for the ligands were determined in control experiments, and these were subtracted from the integrated data before curve fitting was performed.

Time-resolved fluorescence measurements

Many protein interaction assays are designed around protein conjugates whose fluorescence intensity changes between the free and bound states. However, relative changes in the intensity of a fluorophore can be accurately compared by measuring an absolute photo-physical parameter of the fluorescence emission. Four absolute photo physical parameters of the fluorescence emission have been used to

quantify protein interactions *in vitro* and *in vivo*: polarization, lifetime, average energy and quantum yield (Yan and Marriott, 2003). Time-resolved analysis of the emission decay from the excited state of a fluorescent probe provides an opportunity for multiplexing analysis of protein conjugates. Fluorescence lifetime, is an intrinsic property of a fluorophore, which can be considered as a state function as it does not depend on initial perturbation conditions such as wavelength of excitation, duration of light exposure, one- or multiphoton excitation, method of measurement and not affected by photobleaching. In addition, fluorescence lifetime is a parameter largely independent of the fluorescence intensity and fluorophore concentration. Since this process is affiliated with an energetically unstable state, fluorescence lifetime can be sensitive to a great variety of internal factors defined by the fluorophore structure and external factors that include temperature, polarity, and the presence of fluorescence quenchers. A combination of environmental sensitivity and parametric independence mentioned above renders fluorescence lifetime as a separate yet complementary method to traditional fluorescence intensity measurements (Berezin and Achilefu, 2010).

In our fluorescence study lifetime measurements were performed using time correlated single photon counting (TCSPC) machine, model Fluoro Hub (Horiba Jobin Yvon). The samples were excited at 295 nm using a NanoLED pulsed laser. The instrument response function (IRF) was obtained using LudoxTM suspension. The full width at half-maxima (fwhm) of the IRF was 750 ps. The emission decay data at 330 nm were analyzed using the software, DAS6.4, provided with the instrument and decay profile was worked out by using tri exponential fit .

Results and discussion

Effect of divalent cations on the absorption spectra of heme proteins

UV-visible spectroscopy is a routine technique which was used to monitor the structural changes of the proteins like Lb, Mb and Cyt C when complexed with Cu^{2+}, Hg^{2+} or Cd^{2+} . Figure 1A, 1B and 1C represent the UV-visible absorption spectra of Mb with increasing concentration of divalent metal cations in the phosphate buffer (pH 7.0). Addition of cation led to the decrease in absorbance of Mb at 408 nm (soret peak) with isosbestic wavelengths at 432 nm, 423 nm and 414 nm for Cu^{2+}, Hg^{2+} and Cd^{2+} respectively, but no appreciable peak shift (3 nm, 2 nm and 1 nm respectively) was noticed. The relevance for an isosbestic point to occur is that the two species involved are related linearly by stoichiometry, such that the absorbance is invariant for one particular wavelength (Muller, 1994).

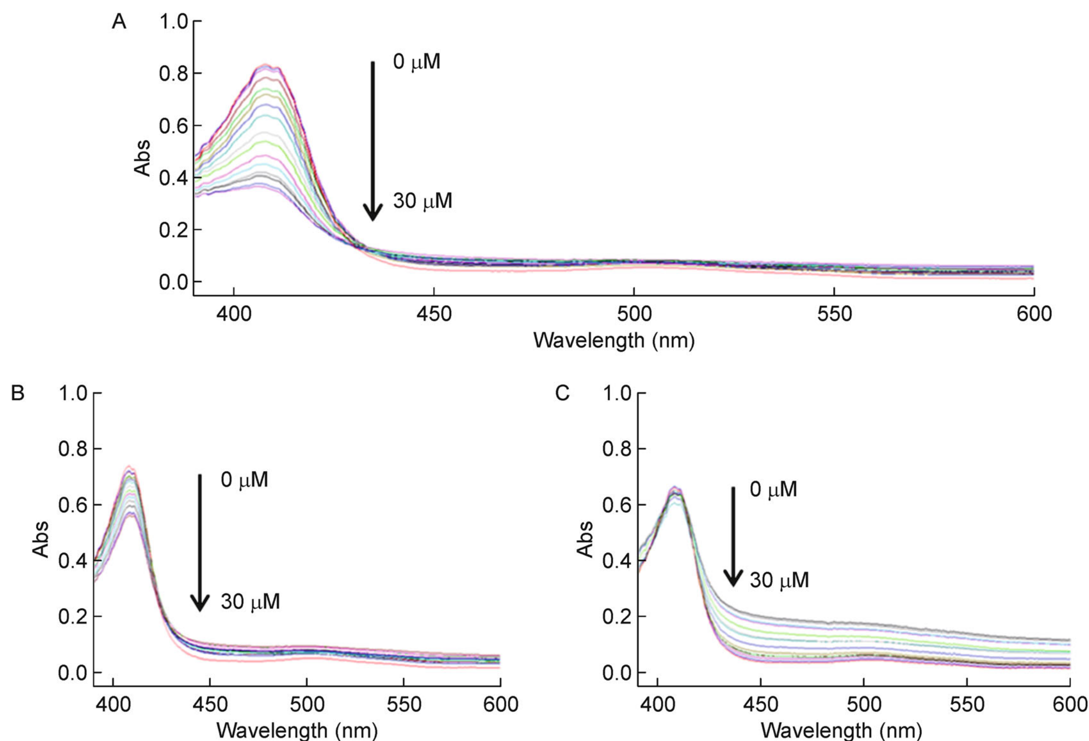

Figure 1 UV-Vis absorption spectra of myoglobin in presence of increasing concentration (0 to 30 μM) of (A) $CuCl_2$ (B) $HgCl_2$ and (C) $CdCl_2$.

An isosbestic point is observed in overlaid spectra when a chromophoric precursor is converted to a product with a different spectrum, so that it is often assumed that an isosbestic point occurs only when the precursor is quantitatively converted to a single product (Berlett et al., 2000). In this study, the presence of isosbestic wavelengths indicate the complex formation between protein and divalent cationic ligands, which was also confirmed by performing ITC and lifetime decay experiments later on. This type of ground state complex is known as dark complex and they may form during static quenching of the protein molecule. Similar dark complex formation was also noticed for Lb-Cu^{2+}, Lb-Hg^{2+} and Lb-Cd^{2+} complex (Figure not provided) . But almost no change in absorption spectra was monitored when Cyt C was titrated with the divalent cations, indicating no ground state complex formation in this case.

Quenching of the intrinsic tryptophan fluorescence by the ligands

Intrinsic fluorescence is a very strong tool to study the conformation and status of the protein in presence of any change in the microenvironment of the protein. Fluorescence quenching refers to any process which decreases the fluorescence intensity of a sample. A variety of molecular interactions can result in quenching, including excited-state reactions, molecular rearrangements, energy transfer, ground-state complex formation, and collisional quenching. The different mechanisms of quenching are usually classified as either dynamic quenching or static quenching (Hua et al., 2005), which can be distinguished by their different dependence on temperature or viscosity, or preferably by lifetime measurements. Lb and Mb both were found to form ground state complexes with Cu^{2+} and Hg^{2+} as was indicated by absorption spectroscopy. In case of Mb-cation interaction, the intensity of Trp fluorescence emission at 331 nm was found to decrease in presence of the divalent cations accompanied with a red shift at emission maximum. Thus static quenching was involved in the process of interaction forming dark complexes. In case of strong quenchers often dark complexes are formed between the fluorophore and the quencher, which corresponds to static quenching mechanism. Static quenching is related to situations where the encounter complex between the fluorophore and the quencher is formed in the ground state of the fluorophore. The dynamic quenching is observed when the fluorophore is excited prior to the encounter complex formation. The difference between the static and dynamic quenching is the sequence of the excitation and complex formation, while the physical basis of the two quenching mechanisms are the same (Mátyus et al., 2006). In our study, apart from static quenching, possibility of involvement of dynamic or collisional interaction was verified by lifetime measurements using TCSPC technique. In Mb, the strong emission maximum of Trp at 331 nm (with excitation at 295 nm) decreased with the increasing concentration of cations and associated red shift of 4 nm for Cu^{2+}, 3 nm for Hg^{2+} and 2 nm for Cd^{2+} were observed. Similar quenching and associated red shifts of 4nm and 5nm for Cu^{2+} and Hg^{2+} respectively were also observed when they reacted with Lb, but no change was observed during the interaction with Cd^{2+}. No any alteration in steady-state fluorescence was noticed during the interaction of divalent cations with cytochrome C. The red shift of emission maximum along with reduction in fluorescence intensity indicates that the interactions of proteins with divalent cations change the environment of tryptophan with probable increase in hydrophilicity from hydrophobic or less hydrophilic environment. The structure of hydrophobic sub domain around Tryptophans was modified and the segment of polypeptide chain changed its conformation to a more extended form due to the ligands. The emission maximum of tryptophans is usually shifted from shorter to longer wavelengths upon protein unfolding, which is consequence of the protein conformational change. The heme proteins Mb and Lb were unfolded with increasing concentrations of divalent cations, and as a result the structure of proteins loosened, forming pores or pathways for ligands to reach the buried tryptophan. Several factors might be responsible for the observed shifts in absorbance and fluorescence of the proteins, and the structural studies alone do not necessarily allow one to discriminate between them (Wachter et al., 1998). A spectral shift to longer wavelengths is expected if the excited state has a larger dipole moment than the ground state, which is almost always the case for π–π^* transitions (Cantor and Schimmel, 1984). During the interaction of divalent cations with the heme proteins, there may be modifications in their three dimensional packing. Thus any micro environmental change in the reaction solution, may influence the emission maximum of Tryptophan, causing a red shift of 2-5 nm where polar solvents decrease the energy separation between π and $\pi*$ levels as a result of preferential stabilization of the excited state. To figure out the mechanism the fluorescence quenching process was analyzed using Stern–Volmer and modified Stern–Volmer plots (Bardhan et al., 2009). Binding of divalent cations to heme proteins was quantitatively estimated by monitoring the changes in the fluorescence intensity during the addition of the metal ions and consequent analysis by S-V plots (Fig. 2A) (Table 1).

The Stern-Volmer constant (K_{sv}) for Mb interacting with Cu^{2+} and Hg^{2+} was 1.69×10^5 mol^{-1} and 4.1×10^4 mol^{-1} respectively whereas K_{sv} value of Lb interacting with Cu^{+2} and Hg^{2+} was 1.16×10^5 mol^{-1} and 1.1×10^4 mol^{-1} respectively (Table 1). From the modified Stern-Volmer plot (Fig. 2B), binding constant of Mb with Cu^{2+} and Hg^{2+} is 7.1×10^3 mol^{-1} and 0.55×10^2 mol^{-1} respectively, indicating that Cu^{2+} binding to Mb and Lb is specific and associated negative free energy change points to the favorable interaction, which seems to be very facile from the thermodynamic aspect (Table 1). The binding stoichiometry is 1 for Cu^{2+} and Mb and 0.91 for Cu^{2+} with Lb . But Hg^{2+} binding for both

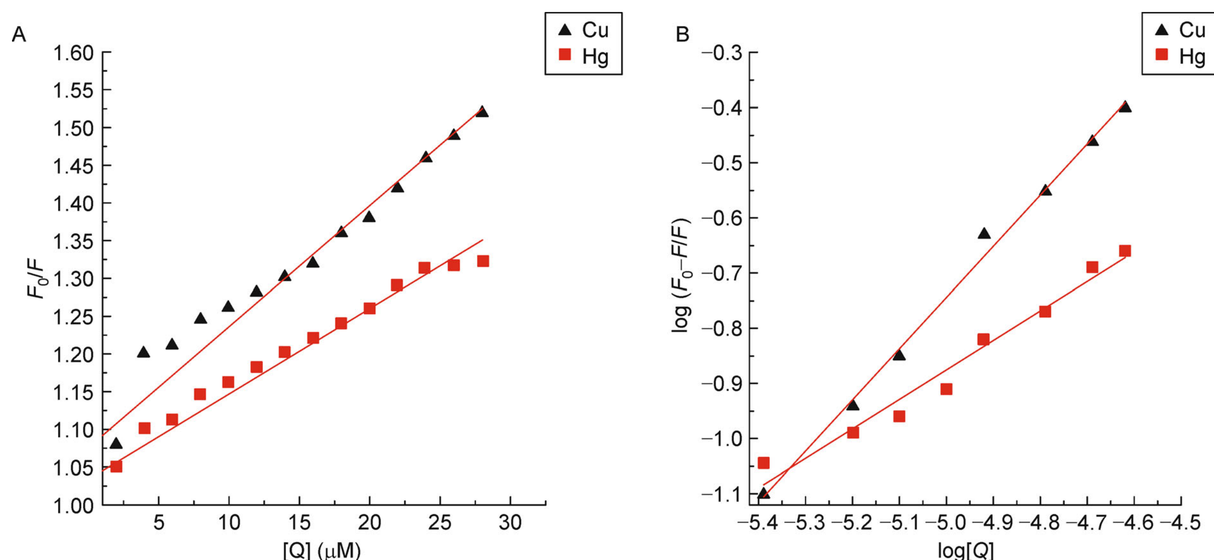

Figure 2 Stern-Volmer plot (A) and modified Stern-Volmer plot (B) for fluorescence quenching of leghemoglobin by $CuCl_2$ and $HgCl_2$.

Table 1 Binding constant and relevant parameters by SV and modified S-V plot in fluorescence quenching

Protein	Metal	SV equation	R^2	K_{sv} (10^6 mol^{-1})	Modified SV $\log[(F_0-F)/F] = \log K_a + n\log[Q]$	R^2	Binding constant(K_a) (mol^{-1})	No. of sites of binding	Free energy(ΔG) ($kJ\cdot mol^{-1}$)
Mb	$CuCl_2$	$Y = 0.0169X + 1$	0.989	0.169	$Y = 0.918X + 3.851$	0.994	7.1×10^3	1	–21.97
	$HgCl_2$	$Y = 0.0041X + 1.075$	0.983	0.041	$Y = 0.521X + 1.740$	0.975	0.55×10^2	0.52	–9.93
Lb	$CuCl_2$	$Y = 0.0160X + 1.075$	0.929	0.16	$Y = 0.914X + 3.385$	0.993	7.07×10^3	0.91	–21.95
	$HgCl_2$	$Y = 0.0011X + 1.030$	0.949	0.011	$Y = 0.521X + 1.740$	0.969	0.56×10^2	0.52	–9.97

Mb and Lb is very similar since binding stoichiometry is always less than 1 (i.e., 0.52). Considering the Stern-Volmer constant and determination of the binding parameters such as the number of sites and the binding constants, steady-state fluorescence method exhibits a strong binding between Mb and and Lb with Cu^{2+} and Hg^{2+}. In the next part of this study TCSPC experiment was performed when decrease in excited state life time established dynamic quenching was associated in the process and we finally inferred that both static and dynamic quenching were involved with the heme protein–divalent cation interaction.

Thermodynamic parameters and nature of binding forces using isothermal calorimetry

The ITC data are very interesting as it can reveal the nature of binding forces which is complementary to spectrocopies method . In general, the binding between ligands and protein molecules include hydrogen bond, electrostatic interaction, hydrophobic interaction and van der walls interaction. According to the nature of entropy and enthalpy changes, we have worked out the model of interaction (Ross and Subramanian, 1981). The energetics of the binding of $CuCl_2$, $HgCl_2$ and $CdCl_2$ with Lb has been plotted in Fig. 3, raw data of Mb has not been shown here due to lack of space. In this study, myoglobin interacts with Cu^{2+} exothermally where as the interaction with Hg^{2+} was endothermic (Table 2).

The entropy change is favorable with Hg^{2+}, but unfavorable in case of Cu^{2+}. Interestingly, in both cases large negative free energy values indicate t the binding to be favorable. Hence, binding of Cu^{2+} with Mb is an enthalpy driven process while Hg^{2+} binding is dominated by entropy contribution (Table 3). The binding stoichiometries for both the metal was around 1. From the table it is clear that binding of Mb with Cu^{2+} is nearly 3000-fold tighter than Hg^{2+} (2.9×10^{-4} mol^{-1} vs. 98 mol^{-1}), indicating that Cu^{2+} binding to Mb is specific. On the contrary, both the metals interact with leghemoglobin endothermally (Table 2, Fig. 3).

It is interesting that in both cases the free energy change is negative indicating favorable interaction with both the metal ions (Table 2). This clearly indicated that metal binding to Lb is largely contributed by positive entropy change. Again positive entropy change also leads to the conclusion that Hg^{2+} binding to Mb or Lb are non-specific. The above data clearly indicated that Cu^{2+} binding to both Mb and Lb are specific. High ΔG value in both cases also revealed that the binding is very facile from thermodynamic aspect. In both cases the binding stoichiometry is nearly 1. For Hg^{2+}, binding with both the proteins is very weak is indicated by very low value of binding constant. The binding is also less than 1. We

Table 2 Thermodynamic parameters of Protein-divalent cation interaction derived by ITC method

Protein	Ligand	Binding constant (mol^{-1})	Enthalpy change (ΔH) (cal/mol)	Entropy change (ΔS) (cal/(mol·deg K))	Free energy change (ΔG) ($kJ\cdot mol^{-1}$)	Remarks
Myoglobin	$CuCl_2$	2.89×10^4	-5.3×10^4	−157	−25.44	Exothermic Enthalpy driven
	$HgCl_2$	98	5.65×10^5	1.9×10^3	−11.36	Endothermic Entropy and enthalpy driven
	$CdCl_2$	1.37×10^4	-3.875×10^4	-0.119×10^3	-0.32×10^4	Exothermic Enthalpy driven
Leghemoglobin	$CuCl_2$	2.39×10^4	6.65×10^4	243	−24.98	Endothermic Entropy and enthalpy driven
	$HgCl_2$	60	5.71×10^6	1.9×10^4	−10.14	Endothermic Entropy and enthalpy driven
	$CdCl_2$	2.26×10^4	5.8×104	0.267×10^3	-2.10×10^4	Endothermic Entropy and enthalpy driven

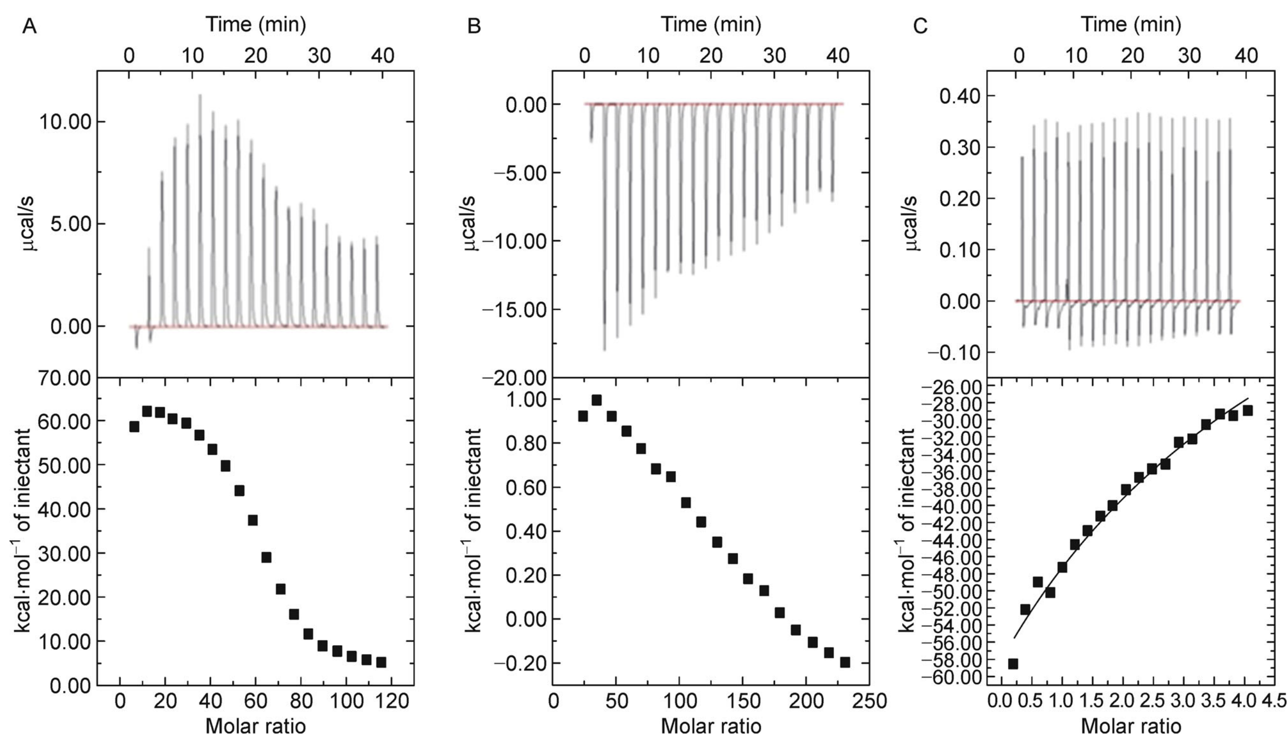

Figure 3 Isothermal titration calorimetric profile for the Lb-divalent interaction with samples at pH 7.0 (0.1M phosphate buffer) at 22°C. The raw data indicates addition of Lb (A) $CuCl_2$, (B) $HgCl_2$, (C) $CdCl_2$.

estimated the binding parameters utilizing spectrofluorimetry and ITC techniques, that differed according to their specific characters which is a regular feature (Basak et al., 2015). In theory, binding can be monitored using ITC because virtually all binding processes are accompanied by uptake or release of heat. The usual differences noticed between parameters determined by ITC and spectroscopic techniques are because ITC records all heat changes. Hence ITC can probe processes that even do not have spectroscopic signatures. Moreover, compared with fluorescence measurements, higher concentrations of probe can be used, which is advantageous for analyzing macromolecule-ligand interactions. This allows us to examine binding of cations in more detail at an extended concentration range of metal ions. Inner filter effects at higher concentration of metal ions interfere with fluorescence measurements but do not pose a problem in calorimeter (Rispens et al., 2008) The binding affinity observed by fluorescence spectroscopy took into consideration the location of quencher, and the fluorophore thus measuring local changes around the fluorophores associated with the optical transition (Nada and Terazima, 2003). Hence to overcome all these shortcomings, we have also authenticated the parameters by ITC measurements that consider overall global changes (Faergeman et al., 1996). Hg^{2+} has got the largest radius within these three divalent cations, so it may be concluded that with increase in radius of heavy metal the

strength of interaction decreased. Moreover, it may be inferred that the structure of Lb is nearly similar to Mb with the same site being used for heavy metal binding.

TCSPC Study for Lb, Mb and Cyt C in presence of divalent cations

We carried out time resolved fluorescence study to explore the nature of binding interactions of divalent cations with heme proteins Lb, Mb and Cyt C. There were three lifetime components in native heme proteins τ_1, τ_2 and τ_3, which contribute depending on the protein's multiple local configurations and changes in the extent of solvent accessibility. Information regarding the conformational state of the metal complexes during an interaction may be derived by analyzing emission spectra of the tryptophan in the complex. Fluorescence lifetime serves as a sensitive parameter for exploring the local environment around a fluorophore and it is sensitive to excited state interaction between the proteins and the probes (Das et al., 2006). The fluorescence lifetime decay profiles of the native proteins Lb, Mb and Cyt C and the protein divalent cation complexes have been presented in Figs. 4, 5 and 6 respectively.

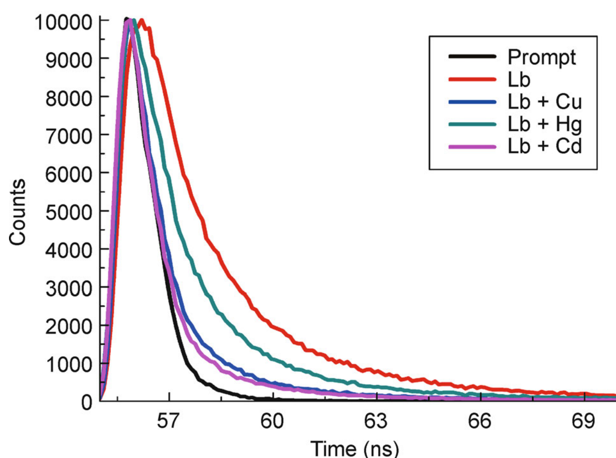

Figure 4 Time-resolved fluorescence decays of native Lb and Lb– metal complex respectively with excitation wavelength of 295 nm. Concentration of Lb: 3 μM, and each metal concentration: 30 μM.

Fluorescence lifetime of tryptophan residues were found to decrease significantly in presence of divalent cations but differentially for Lb, Mb and Cyt C. Average excited state life time of Trp residue in Lb was decreased from 2.57 ns to 1.28ns, 1.87ns and 2.01ns due to the interaction with Cu^{2+}, Hg^{2+} and Cd^{2+} respectively (Table 3).

Similarly the excited state lifetime of tryptophan residue in Mb was 2.32 ns and was reduced to 1.59 ns, 1.87 ns and 2.17 ns due to the interaction with Cu^{2+}, Hg^{2+} and Cd^{2+} respectively with no any significant change for the last cation. The lifetime of tryptophan residues in Cyt C was

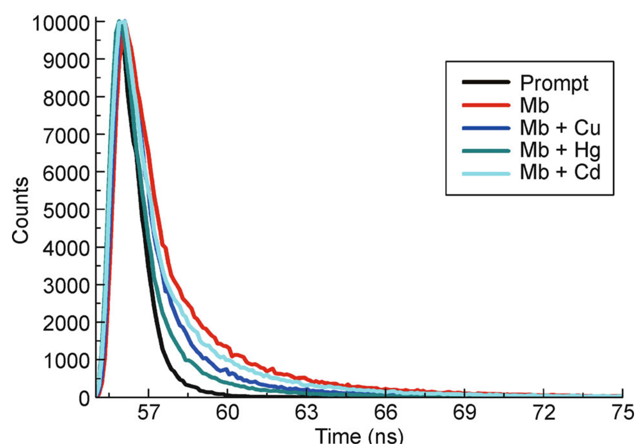

Figure 5 Time-resolved fluorescence decay of native Mb and Mb – metal complex respectively with excitation wavelength of 295 nm. Concentration of Mb: 3 μM, and each metal concentration: 30 μM.

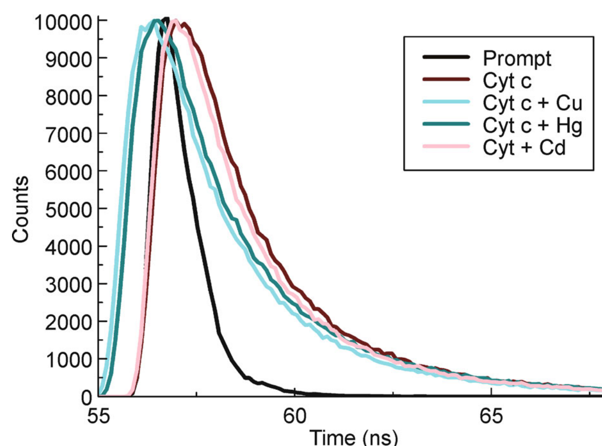

Figure 6 Time-resolved fluorescence decays of native Cyt C and Cyt C – metal complex respectively with excitation wavelength of 295 nm. Concentration of Cyt C: 3 μM, and each metal concentration: 30 μM.

2.12 ns, and observed to be 1.70 ns, 1.86 ns and 2.02 ns during the interaction with Cu^{2+}, Hg^{2+} and Cd^{2+} indicating very small changes. It is worth mentioning here that no changes in absorption values or steady-state fluorescence intensity was noticed during the interaction of divalent cations toward Cyt C though very small changes was noticed in excited state lifetime. These finding possibly reveal that the metal complexes of Cyt C are extremely transient and unstable which is beyond the preview of any kind of steady-state measurement. In this context, the work of Gourion-Arsiquand et al. (2005) should be referred, where interaction of Cyt C with Cd^{2+} was studied using FTIR coupled to an ATR Microdialysis Setup and NMR Spectroscopy. Due to no change or less drastic conformational change of the Cyt C after metal binding no significant alteration in steady-state

Table 3 Fluorescence life time decay profile of protein-divalent cation interaction by TCSPC method

Protein		α_1	τ_1	α_2	τ_2	α_3	τ_3	Average (τ)	χ^2
Leghemoglobin	**Native**	0.12	0.30	0.36	2.0	0.52	5.43	2.57	1.21
	$CuCl_2$	0.10	0.13	0.21	0.82	0.69	2.90	1.28	1.40
	$HgCl_2$	0.14	0.60	0.27	1.47	0.59	3.55	1.87	1.13
	$CdCl_2$	0.16	0.40	0.32	1.73	0.52	3.90	2.01	1.11
Myoglobin	**Native**	0.06	0.08	0.24	1.91	0.70	4.99	2.32	1.27
	$CuCl_2$	0.05	0.06	0.15	1.33	0.80	3.38	1.59	1.30
	$HgCl_2$	0.07	0.09	0.210	1.80	0.72	3.73	1.87	1.20
	$CdCl_2$	0.07	0.09	0.20	1.40	0.73	5.03	2.17	1.33
Cytochrome c	**Native**	0.16	0.70	0.28	1.99	0.56	3.98	2.12	1.08
	$CuCl_2$	0.13	0.25	0.29	1.60	0.58	3.27	1.70	1.30
	$HgCl_2$	0.10	0.22	0.26	1.40	0.64	3.97	1.86	1.04
	$CdCl_2$	0.09	0.14	0.180	1.80	0.73	4.14	2.02	1.23

spectra was observed. This crucial observation became possible only applying powerful techniques like IR spectroscopy due to their vibrational nature.

According to earlier report, the underlying mechanism of the interaction between heavy metal binding sites might be related to the structure and function of the heme protein involving both the heme-heme and mercapto-mercapto interaction which vanishes when the molecule was denatured (Murayama, 1959). Copper has been found to intensely combine with protein component and ferroporphyrin component in cytochrome C (Wang et al., 2006). But, in contrast to Hb, Mb has only one Cysteine residue and Cyt C or Lb does not have any Cysteine residue, so the involvement of Cyst residue or sulfhydryl group leading to cross-linking and consequent conformational linkage is not possible in this specific case. Most of the natural metalloporphyrins are of regular type, i.e. their metal centers are located within the plane of the macrocyclic ligand as a consequence of their fitting size (Giovannetti and Uddin, 2012). The cationic radii are in the range of 55–80 pm corresponding to the sphere in the porphyrin core surrounded by the four pyrrolic nitrogens (Kahn and Bruice, 2003). If, however, the ionic radius of the metal ions is too large (over ca. 80–90 pm) to fit into the hole in the center of the macrocycle, they are located out of the ligand plane, distorting it forming sitting-atop (SAT) metalloporphyrins that are characterized by special properties (Liao et al., 2006; Walker et al., 2010).These complexes are kinetically labile and display characteristic structural and photo induced properties that strongly deviates from those of the regular metalloporphyrins. In our study, considering the ionic radius of the three cations, Hg^{2+} (116 pm) is the largest compared to Cu^{2+} (91 pm) and Cd^{2+} (99 pm) (Pauling, 1960), but all of them have radius greater than 80 pm. Thus none of them is capable to fit in the center of the macrocycle and will be located out of the ligand plane, distorting it forming sitting-atop (SAT) metalloporphyrins, which are deviated from their regular characteristics as mentioned above. So iron cation in porphyrin core cannot be replaced by these transition elements like Hg^{2+}, Cu^{2+} or Cd^{2+}. On the other hand, steady-state and time resolved fluorescence decay studies evidenced that, both static and dynamic quenching were involved in these protein-cation interaction, when ground state complex formation as well as bimolecular collisional encounter at excited state also take place. Thus, we may infer that not only the presence of tryptophan residue or the conserved sequences, or the existence of porphyrin ring in heme proteins but the environment provided by the globin moiety and the heme pocket in these monomeric heme proteins, combined with the optimum size of the cation radii may create a crucial folding or topology needed for the complexation of metal ions in the protein . In many aspects tryptophan is a special amino acid having the largest surface area and it is a highly preformed component of residue clusters in protein structures (Heringa and Argos, 1991) .But in these specific cases though alteration in tryptophan emission points the involvement of the micro environment of this aromatic residue in the interaction but it is not the only determining factor. Stereo specific interactions observed in tryptophan and heme environment have relevance in understanding these interactions. The accessible surface area of tryptophan decreases exponentially with the number of residues around it, and this relationship may also provide a way to access the efficiency of packing around any tryptophan residue (Samanta et al., 2000).

Conclusion

The mechanism of binding of the divalent cations to heme proteins was investigated using UV-visible, steady-state, and fluorescence lifetime measurements. Our results indicate that disturbances of metal homeostasis in plants and organisms, may lead to the formation of metal complexes resulting alteration in the native structure of the protein, which in consequence would generate unfolded, pathogenic protein structures in cells disturbing normal functioning. The fluorescence of the heme proteins (Mb and Lb) evidenced a

red (bathochromic) shift along with a reduction in the fluorescence intensity and fluorescence lifetime upon binding with the divalent cations. In this study, we found, transition metal ions Cu^{2+} and Hg^{2+} easily formed metal complexes with Lb and Mb whereas Cd^{2+} formed only transient complexes which possibly lacks stability. Cyt C, though similar type of monomeric heme protein, we could not find any stable metal complexes with Cu^{2+}, Hg^{2+} or Cd^{2+} .Thus, it points to the fact that the presence of tryptophan and other specific conserved residues in heme proteins or, the porphyrin prosthetic group is not the sole responsible target for the transition metal complexes to be formed. Rather, the selective binding of the divalent cations seems to be associated with multiple factors like optimum size of cationic radii, the microenvironment of tryptophan residue and the heme pocket, some specific domain or specific topology in the proteins. Any of these factors or combined effect of them may be the crucial components for the formation of protein-metal complexes, since small ligands may bind to depressions on or near the surface of proteins or in the clefts between the domains. This work also unfolds the fact that Lb has very similar conformation like Mb but remarkably different than Cyt C, though each of them being monomeric heme protein.

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Compliance with ethics guidelines

Pijush Basak , Tanay Debnath , Rajat Banerjee and Maitree Bhattacharyya declare that they have no conflict of interest. This article does not contain any studies with human or animal subjects performed by any of the authors

References

Arias-Moreno X, Abian O, Vega S, Sancho J, Velazquez-Campoy A (2011). Protein-cation interactions: structural and thermodynamic aspects. Curr Protein Pept Sci, 12(4): 325–338

Bardhan M, Mandal G, Ganguly T J (2009). Steady state, Time resolved and Circular dichroism spectroscopic studies to reveal the nature of interactions of zinc oxide nanoparticles with transport protein Bovine Serum Albumin and to monitor the possible protein conformational changes. Appl Phys (Berl), 106(3): 34701–34705

Basak P, Bhattacharyya M (2013). Intrinsic tryptophan fluorescence and related energy transfer in Leghemoglobin isolated from *Arachis hypogeal.* Turkish. J Biochem, 38: 9–13

Basak P, Pattanayak R, Bhattacharyya M (2015). Transition metal induced conformational change of heme proteins. Spectrosc Lett, 48 (5): 324–330

Berezin M Y, Achilefu S (2010). Fluorescence lifetime measurements and biological imaging. Chem Rev, 110(5): 2641–2684

Berlett B S, Levine R L, Stadtman E R (2000). Use of isosbestic point wavelength shifts to estimate the fraction of a precursor that is converted to a given product. Anal Biochem, 287(2): 329–333

Cantor C R, Schimmel P R (1984). Biophysical Chemistry Part II: Techniques for the Study of Biological Structure and Function. New York: W.H.Freeman and Company, 386–398

Das P, Mallik A, Halder B, Chakraborty A, Chattopadhyay N (2006). Effect of nanocavity confinement on the rotational relaxation dynamics: 3-acetyl-4-oxo-6,7-dihydro- 12 H indolo- [2, 3- a] quinolizine in micelles. J Chem Phys, 125(4): 044516

Dickerson R E, Timkovich R (1975). The Enzymes, (P. Boyer Ed.). New York: Academic Press

Faergeman N J, Sigurskjold B W, Kragelund B B, Andersen K V, Knudsen J (1996). Thermodynamics of ligand binding to acyl-coenzyme A binding protein studied by titration calorimetry. Biochemistry, 35(45): 14118–14126

Giovannetti R, Uddin J (2012). The Use of Spectrophotometry UV-Vis for the Study of Porphyrins, InTech, 95–96, ISBN: 978–953–51–0664–7

Gourion-Arsiquaud S, Chevance S, Bouyer P, Garnier L, Montillet J L, Bondon A, Berthomieu C (2005). Identification of a Cd^{2+}- and Zn^{2+}-binding site in cytochrome c using FTIR coupled to an ATR microdialysis setup and NMR spectroscopy. Biochemistry, 44(24): 8652–8663

Harbury H A, Loach P A (1960). Oxidation-linked proton functions in heme octa- and undecapeptides from mammalian cytochrome c. J Biol Chem, 235: 3640–3645

Heringa J, Argos P (1991). Side-chain clusters in protein structures and their role in protein folding. J Mol Biol, 220(1): 151–171

Hua Y J, Liua Y I, Zhanga L X, Zhaoa R M, Qua S S (2005). Studies of interaction between colchicine and bovine serum albumin by fluorescence quenching method. J Mol Struct, 750(1-3): 174–178

Kadish K M, Smith K M, Guilard R (2010). Handbook of Porphyrin Science. World Scientific Publishing: Singapore

Kahn K, Bruice T C (2003). Comparison of reaction energetics and leaving group interactions during the enzyme-catalyzed and uncatalyzed displacement of chloride from haloalkanes. J Phys Chem B, 107(28): 6876–6685

Kang J, Liu Y, Xie M X, Li S, Jiang M, Wang Y D (2004). Interactions of human serum albumin with chlorogenic acid and ferulic acid. Biochim Biophys Acta, 1674(2): 205–214

Lakowicz J R (2006). Principles of Fluorescence Spectroscopy, 3rd ed. New York: Plenum Press, 277–285

Lehrer S S (1971). Solute perturbation of protein fluorescence. The quenching of the tryptophyl fluorescence of model compounds and of lysozyme by iodide ion. Biochemistry, 10(17): 3254–3263

Liao M S, Watts J D, Huang M J (2006). DFT/TDDFT study of lanthanide(III) mono- and bisporphyrin complexes. J Phys Chem A, 110(48): 13089–13098

Marchon J C, Mashiko T, Reed C A (1982). Electron-Transport and Oxygen Utilization. (C.H.O. Ed.) North Holland, New York: Elsevier

Mata L, Sanchez L, Calvo M (1997). Interaction of mercury with human and bovine milk proteins. Biosci Biotechnol Biochem, 61(10): 1641–1645

Mátyuss L, Szöllosi J, Jenei A (2006). Steady-state fluorescence quenching applications for studying protein structure and dynamics. J Photochem Photobiol B, 83(3): 223–236

Muller P (1994). Glossary of terms used in physical organic chemistry. Pure Appl Chem, 66(5): 1077

Murayama M (1958). Titrable sulphahydryl groups of Hemoglobin C and fetal Hemoglobin at 0° and 38°. J Biol Chem, 230: 163–168

Murayama M (1959). On the nature of the interaction between binding sites for heavy metals (mercapto-mercapto interactions) in normal human hemoglobin. J Biol Chem, 234: 3158–3162

Murphy C B, Zhang Y, Troxler T, Ferry V, Martin J J, Jones W E (2004). Probing Förster and Dexter energy-transfer mechanisms in fluorescent conjugated polymer chemosensors. J Phys Chem, 108(5): 1537–1543

Nada T, Terazima M (2003). A novel method for study of protein folding kinetics by monitoring diffusion coefficient in time domain. Biophys J, 85(3): 1876–1881

Ordway G A, Garry D J (2004). Myoglobin: an essential hemoprotein in striated muscle. J Exp Biol, 207(Pt 20): 3441–3446

Pauling L (1960). The Nature of the Chemical Bond (3rd Edn.), Ithaca, NY: Cornell University Press

Pinto M R, Schanze K S (2004). Amplified fluorescence sensing of protease activity with conjugated polyelectrolytes. Proc Natl Acad Sci USA, 101(20): 7505–7510

Raphael A L, Gray H B (1991). Semisynthesis of axial-ligand (position 80) mutants of cytochrome c. J Am Chem Soc, 113(3): 1038–1040

Rispens T, Lakemond C M M, Derksen N I, Aalberse R C (2008). Detection of conformational changes in immunoglobulin G using isothermal titration calorimetry with low-molecular-weight probes. Anal Biochem, 380(2): 303–309

Ross P D, Subramanian S (1981). Thermodynamics of protein association reactions: forces contributing to stability. Biochemistry, 20(11): 3096–3102

Samanta U, Pal D, Chakrabarti P (2000). Environment of tryptophan side chains in proteins. Proteins, 38(3): 288–300

Takeda K, Hachiya K (2002). Interaction of Protein with Ionic Surfactant, Part I: Marcel Dekker; New York

Wachter R M, Elsliger M A, Kallio K, Hanson G T, Remington S J (1998). Structural basis of spectral shifts in the yellow-emission variants of green fluorescent protein. Structure, 6(10): 1267–1277

Walker V E, Castillo N, Matta C F, Boyd R J. (2010). The Effect of multiplicity on the size of iron(II) and the structure of iron(ii) porphyrins. J Phys Chem A, 114:10315–10319

Wang J, Guo D, Yuan X (2006). Influence of copper on the interaction between cytochrome c and sulfite *in vitro*. J Biochem Mol Toxicol, 20 (5): 255–258

Wuthrich R Q (1985). Amino acid sequence, haem iron coordination geometry and functional properties of mitochondrial and bacterial c-type cytochromes. Rev Biophys, 18(02): 111–134

Yan Y, Marriott G (2003). Analysis of protein interactions using fluorescence technologies. Curr Opin Chem Biol, 7(5): 635–640

Zaidi N, Ahmad E, Rehan M, Rabbani G, Ajmal M R, Zaidi Y, Subbarao N, Khan R H (2013). A comprehensive insight into binding of hippuric acid to human serum albumin: A study to uncover its impaired elimination through hemodialysis. J Phys Chem B, 117: 2595–2604

Zhang Y Z, Xiang X, Mei P, Dai J, Zhang L L, Liu Y (2009). Spectroscopic studies on the interaction of Congo Red with bovine serum albumin. Spectrochim Acta A Mol Biomol Spectrosc, 72(4): 907–914