A comparative spectroscopic and calorimetric investigation of the interaction of amsacrine with heme proteins, hemoglobin and myoglobin

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The binding of the anilido aminoacridine derivative amsacrine with the heme proteins, hemoglobin, and myoglobin, was characterized by various spectroscopic and calorimetric methods. The binding affinity to hemoglobin was \((1.21 \pm .05) \times 10^5 \text{ M}^{-1}\), while that to myoglobin was three times higher \((3.59 \pm .15) \times 10^5 \text{ M}^{-1}\). The temperature-dependent fluorescence study confirmed the formation of ground-state complexes with both the proteins. The stronger binding to myoglobin was confirmed from both spectroscopic and calorimetric studies. The binding was exothermic in both cases at the three temperatures studied, and was favored by both enthalpy and entropy changes. Circular dichroism results, three-dimensional (3D) and synchronous fluorescence studies confirmed that the binding of amsacrine significantly changed the secondary structure of hemoglobin, while the change in the secondary structure of myoglobin was much less. New insights, in terms of structural and energetic aspects of the interaction of amsacrine with the heme proteins, presented here may help in understanding the structure-activity relationship, therapeutic efficacy, and drug design aspects of acridines.

**Keywords:** hemoglobin; myoglobin; ligand amsacrine; fluorescence; calorimetry

1. Introduction

Hemoglobin and myoglobin are two iron-containing heme proteins associated with oxygen transport and oxygen storage processes, respectively (Ordway & Garry, 2004; Thomas & Lumb, 2012). Hemoglobin (Hb) is a protein molecule in red blood cells responsible for oxygen carrying in the vascular system of vertebrates (McLellan & Walsh, 2004). Hb is a tetrameric protein of molecular mass 64.5 kDa (Kim-Shapiro & Kim-Shapiro, 2009). Hb consists of two \(\alpha\)-chains of 141 amino acids and two \(\beta\)-chains of 146 amino acids (Perutz, 1963). There are four heme groups located in the crevices near the surface of Hb, each attached to one polypeptide chain. Myoglobin (Mb), on the other hand, is a monomeric heme protein with a relatively small mass (17 kDa), found mainly in muscle tissues, where it serves as an intracellular storage site for oxygen (Ordway & Garry, 2004; Wittenberg & Wittenberg, 2003). During oxygen deprivation, oxygen is released for use by muscle mitochondria for oxygen-dependent ATP synthesis. The heme group of Mb is packed within its predominantly \(\alpha\)-helical secondary structure and is coordinated by a histidine residue as the fifth ligand of the heme’s central Fe ion (Evans & Brayer, 1990). Since Hb and Mb are two important functional proteins for reversible oxygen carrying and storage, it is very important to study the influence of drugs on the structure of these two proteins. It is known that the drugs are distributed to active sites based on their affinity to the plasma proteins. Although Hb and Mb are not strictly carrier proteins, Hb will be available in the plasma on hemolysis and under some diseased conditions. Myoglobin, on the other hand, is found in the blood stream after muscle injury. The presence of Hb and Mb in the blood plasma is thus diagnostically relevant in discerning the distribution, metabolism, and toxic action of many small molecules by interacting with them. Therefore, investigations on the interaction with these heme proteins are of great importance in terms of understanding the pharmacological action of drugs. A number of studies on Hb-ligand interaction have been published recently. Nevertheless, studies on the binding of ligands to Mb are scarce.

Amsacrine (Figure 1) is an anilino-aminoacridine derivative used in front line therapy to treat acute leukemias, Hodgkin’s, and non-Hodgkin’s lymphomas (Jehn & Heinemann, 1991; Kell, 2006; Verma et al., 2010). The drug structure (Figure 1) comprises an acridine moiety coupled to a 4′-amino-methanesulfon- m-anisidine head group. The anticancer activity of amsacrine appears...
to stem from its ability to intercalate into duplex DNA, stabilize the ternary topoisomerase II–DNA complex and cause DNA double-strand breaks (Errington et al., 1999; Neidle & Waring, 1993).

In this manuscript, we have examined the nature of interaction of the anilido aminoacridine derivative amsacrine with these heme proteins using a combination of spectroscopy and calorimetry techniques to understand its pharmaceutical utility and molecular basis of drug action.

2. Experimental

2.1. Materials

Human methemoglobin (CAS Number: 9008-02-0, \( M = 64,500 \) Da purity \( \geq 85\% \)), equine skeletal muscle myoglobin (CAS Number: 100684-32-0, \( M = 17600 \) kDa, purity \( \geq 90\% \)), amsacrine (CAS Number: 54301-15-4, \( M = 429.92 \) Da, purity \( \geq 98\% \)), and 1,8-anilinonaphthalenesulfonic acid (CAS Number: 82-76-8, purity \( \geq 90\% \)), and 188 mM HCl (Antonini & Brunori, 1971), 12 mM \( \text{NaCl} \) (Antonini & Brunori, 1971), 188 mM \( \text{NaCl} \) at 408 nm for Mb (Antonini & Brunori, 1971; Castro-Forero, Jiménez, López-Garriga, & Torres-Lugo, 2008) and 12 mM \( \text{NaCl} \) at 434 nm for amsacrine (Bourdouxhe-Housiaux, Colson, Houssier, Waring, & Bailly, 1996). No deviation from Beer’s law was observed for the concentration range of amsacrine used in this study. All other chemicals used were of analytical grade. The buffer solution was prepared from deionized and double-distilled water and filtered through membrane filters of pore size \( 0.22 \mu \text{m} \).

2.2. Instruments and methods

2.2.1. Absorption and fluorescence spectral studies

The absorbance spectra were measured at 298.15 \( \pm 1 \) K on a Jasco V660 double-beam double-monochromator spectrophotometer (Jasco International Co., Hachioji, Japan) in 1-cm path length quartz cuvettes. Standard drug–protein titration methodologies, described in detail earlier, were followed (Chatterjee & Suresh Kumar, 2014; Hazra, Hossain, & Suresh Kumar, 2013; Hazra & Suresh Kumar, 2014).

Steady-state fluorescence measurements were performed on a Shimadzu RF-5301PC (Shimadzu Corp., Kyoto, Japan) fluorescence spectrometer in fluorescence-free quartz cuvettes of 1-cm path length (Hossain, Khan, & Suresh Kumar, 2011; Hazra et al., 2013; Basu & Suresh Kumar 2015a). The experiments were performed keeping excitation and emission bandwidth of 5 nm. The sample temperature was maintained at 298.15 \( \pm 1 \) K using an Eyela UniCool U55 water bath (Tokyo Rakakikai Co. Ltd., Tokyo, Japan). The fluorescence quenching of the protein was measured using the excitation wavelength of 295 nm for the intrinsic tryptophan fluorophore. Excitation wavelength was 434 nm for amsacrine.

Temperature-dependent fluorescence spectral studies were performed in a Hitachi F4010 unit equipped with a circulating water bath. Synchronous fluorescence spectra were measured in the excitation range of 220–380 nm keeping \( \Delta \lambda \) set at 15 and 60 nm, respectively. Correction of the fluorescence intensities for the absorption of exciting light and reabsorption of the emitted light was applied to decrease the inner filter effect (Hazra & Suresh Kumar, 2014).

Three-dimensional (3D) fluorescence spectroscopy experiments were performed on a PerkinElmer LS55 fluorescence spectrometer (PerkinElmer, Inc., USA). The initial wavelength was set at 200 nm and extended to 340 nm with an increment of 10 nm for each scan. The fluorescence emission spectra of Hb were measured in the wavelength range of 270–500 nm.

2.2.2. Circular dichroism spectra and FTIR studies

Circular dichroism (CD) spectra were acquired on a Jasco J815 unit (Jasco International Co.) equipped with a Jasco temperature controller (PFD 425L/15) as reported (Hazra & Suresh Kumar, 2014; Hazra et al., 2013). A Peltier cell holder and temperature controller PFD 425 L/15 were used to maintain the cuvette temperature at 298.15 K. A scan speed of 20 nm/min, bandwidth of 1.0 nm and sensitivity of 100 milli degrees were applied. Five successive scans were performed and averaged to improve the signal-to-noise ratio and smoothed within permissible limits by the

Figure 1. Chemical structure of amsacrine.
software. The molar ellipticity values were expressed in terms of the mean residue molar ellipticity [θ], in units of deg cm² dmol⁻¹. FTIR measurements were performed on a Bruker Tensor 27 FT-IR spectrometer (BRUKER).

2.2.3. Optical thermal melting and differential scanning calorimetry studies

Absorbance vs. temperature curves (melting profiles) of heme proteins and their complexes with amsacrine were obtained on Shimadzu Pharmaspec 1700 unit having a Peltier-controlled TMSPC-8 model accessory (Shimadzu Corp., Kyoto, Japan) as described in details earlier (Hazra & Suresh Kumar, 2014). Temperature-dependent transitions of the hemoglobin and myoglobin as excess heat capacities were measured in a Microcal VP-DSC unit (MicroCal, Inc., Northampton, MA, USA) as described previously (Basu & Suresh Kumar, 2014; Michnik, Drzazga, Kluczewszka, & Michalik, 2005).

2.2.4. Isothermal titration calorimetry

Isothermal titration calorimetry (ITC) experiments were performed in a MicroCal VP-ITC unit (MicroCal Inc.). The proteins and amsacrine solutions were thoroughly degassed on the MicroCal’s Thermovac unit before loading to avoid air bubble formation during the course of the titration. Aliquots of the degassed amsacrine solution were injected from a pre-programmed rotating syringe (290 rpm) into the isothermal calorimeter chamber containing the degassed heme protein solution (1.4235 mL). The area under each heat burst curve was determined by integration using the Origin 7.0 software to give a measure of the heat associated with the injection. The heat associated with each amsacrine–buffer mixing was subtracted from the corresponding heat of amsacrine–protein reaction to give the heat of amsacrine–protein binding. The heat of dilution of injecting the buffer into the protein solution was found to be negligible. The resulting corrected injection heats were plotted as a function of molar ratio. A model for one set of binding sites provided the binding affinity (Kₐ), the binding stoichiometry (N), and the standard molar enthalpy of binding (Δf°). The standard molar Gibbs energy change (ΔG°) and the standard molar entropic contribution to the binding (∆TS°) were subsequently calculated from standard relationships (Damian, 2013; Giri & Suresh Kumar, 2009). The ITC unit was periodically calibrated and verified with water–water dilution experiments as per the criteria of the manufacturer.

3. Results and discussion

3.1. UV–visible spectroscopic analysis of amsacrine–protein complexes

Amsacrine has a characteristic UV–visible absorption spectrum (Supplementary Figure S1) in the 200–600 nm region with a sharp peak around 255 nm and a board peak around 434 nm. The absorption spectra of Hb and Mb have two major peaks in the UV–vis region with maxima around 195 nm (π → π* transition of >C=O group of amino acids) and 405 nm (Soret band), respectively. The change in the absorption spectra of Hb and Mb in the presence of amsacrine was monitored to understand the interaction phenomena. In the presence of increasing concentration of amsacrine, a decrease in the intensity of both bands was observed, the changes being higher for the band near 195 nm. Typical absorbance spectral changes in the spectra of Hb and Mb in the presence of amsacrine are presented in Figure 2(A) and (B). In the inset, the changes in the 406 nm Soret bands in the presence of amsacrine are presented. Pronounced hypochromic and bathochromic effects were observed in the former band for both Hb and Mb and the change was more pronounced in the case of Mb; only hypochromic effect was observed in the Soret band, suggesting complex formation in the ground state. The spectral titration data were analyzed by Benesi–Hildebrand plot to determine the equilibrium constants (Benesi & Hildebrand, 1949). Higher binding affinity value was observed for the interaction of amsacrine with Mb (1.05 ± .06 × 10⁵ M⁻¹) compared to Hb (7.45 ± .15 × 10⁴ M⁻¹).

3.2. Fluorescence spectral studies

The intrinsic fluorescence property of proteins has proved to be a useful probe of protein structure, function, and dynamics. The intrinsic fluorescence of Hb and Mb arise essentially from the, tryptophan (Trp), tyrosine (Tyr), and phenylalanines (Phe) residues present in the polypeptide chain. Myoglobin possesses two Tyr, two Trp, and seven Phe residues (Kendrew, 1963). The tetrameric Hb contains a total of six Trp residues, three Trp residues each in the two αβ dimers as α-Trp14, β-Trp15, and β-Trp37 (Venkatesh rao & Manoharan, 2004) and five Tyr residues in each αβ dimer as α-Tyr24, α-Tyr42, α-Tyr140, β-Tyr34, and β-Tyr144 (Mueser, Rogers, & Arnone, 2000). The primary fluorescence emission of the Hb molecule has been identified to be from the β-Trp37 residue located at the αβ2 interface (Alpert, Jameson, & Weber, 1980). Furthermore, it is also known that the emission maximum of tryptophans is more sensitive to the local environment than that of the tyrosines. So, a study of the change in intrinsic fluorescence of heme proteins in the presence of the amsacrine was performed to gather information about the local environment of the Trp moiety on interaction. Both Hb and Mb showed emission maximum at 328 nm when excited at 295 nm. It was observed that amsacrine quenched the fluorescence intensity of both proteins. The quenching mechanism could be the result from inner filter effect, collisional quenching (dynamic), or binding-related
Dynamic and static quenching can be differentiated conventionally by their differential responses towards temperature. So a temperature-dependent study can easily differentiate this. Static quenching is caused by ground-state complex formation with the fluorophore. Therefore, increasing temperature will decrease the quenching constant ($K_{sv}$) due to declining stability of the ligand–protein complex. On the other hand, the diffusion coefficients are larger at higher temperature and hence the value of $K_{sv}$ will increase with the increase in temperature for systems where dynamic quenching prevails.

In order to elucidate the quenching mechanism in the interaction of amsacrine with Hb and Mb, temperature-dependent fluorescence spectral experiments were carried out at three different temperatures, 288.15, 298.15, and 308.15 K, and the data were analyzed by classical Stern–Volmer equation.

$$
\frac{F_o}{F} = 1 + K_q \tau_o [Q] = 1 + K_{sv} [Q]
$$

Here, $F_o$ and $F$ are the fluorescence intensities in the absence and presence of the quencher, $K_q$ is the quenching rate constant and is equal to $K_{sv}/\tau_o$, $K_{sv}$ is the Stern–Volmer quenching constant, $\tau_o$ is the average life time of the protein in the absence of quencher which is of the order of $10^{-8}$ s (Lakowicz & Weber, 1973) and $[Q]$ is the concentration of the free quencher, respectively. The values of $K_q$ and $K_{sv}$ are listed in Table 1. The Stern–Volmer quenching constant is inversely correlated with temperature and the values of $K_q$ are much larger than the maximum scattering collision quenching constant ($2 \times 10^{10}$ L mol$^{-1}$ s$^{-1}$), suggesting a static quenching mechanism for the amsacrine–protein complexation reaction.

The fluorescence data were also analyzed by Lineweaver–Burk equation. Here, also a decreasing trend in the values of the quenching constants (Table 1) was obtained and this further confirmed static quenching mechanism operating in the complexation.

### 3.3. Elucidation of the binding affinity

The equilibrium binding constant ($K_A$) and the number of binding sites ($n$) for the interaction of amsacrine with heme proteins were calculated using the following equation (Abou-Zied & Al-Shihi, 2008)

$$
\log \left( \frac{F_o - F}{F} \right) = \log K_A + n \log [Q]
$$

Here, $K_A$ is the binding constant to a binding site and $n$ is the number of binding sites per protein. The values of $K_A$ and $n$ obtained from the analysis of the data are presented in Table 1. The decreasing trend of the binding constant with increasing temperature suggests that the stability of the amsacrine–protein complex is weakened with increasing temperature. The values of $n$ approximately equal to 1 indicated the existence of a single binding site. It is also evident from the bar graph (Figure 3) that amsacrine has higher affinity to Mb compared to Hb.

### 3.4. Conformational aspects of the interaction

Synchronous fluorescence studies were performed to explore the microenvironment of amino acid residues via measurement of the emission wavelength shift (Congdon, Muth, & Splittgerber, 1993). We already know that the synchronous fluorescence spectra of the heme proteins provide the characteristic information for the Tyr and Trp residues when the wavelength interval $\Delta \lambda$ ($\Delta \lambda = \lambda_{em} - \lambda_{ex}$) is fixed at 15 and 60 nm, respectively. The shift in the position of fluorescence emission maxima corresponds to changes of the polarity around the chromophore molecule. A blue shift of $\lambda_{max}$ means that
Table 1. Binding data derived for amsacrine binding to Hb and Mb from spectrofluorometric studies at different temperatures.

| Temp (K) | Stern–Volmer quenching constant ($K_{SV} \times 10^{-4}$ M$^{-1}$) | Quenching rate constant ($K_q \times 10^{-12}$ M$^{-1}$ s$^{-1}$) | Apparent binding constant ($K_d \times 10^{-5}$) M$^{-1}$ | Correlation coefficient for $K_d$ | Lineweaver–Burk constant ($K_{LB} \times 10^{-4}$ M$^{-1}$) |
|----------|---------------------------------------------------------------|---------------------------------------------------------------|---------------------------------------------------------|----------------------------------|----------------------------------------------------------|
| Hb       |                                                               |                                                               |                                                         |                                  |                                                          |
| 288.15   | 6.87±.08                                                      | 6.87                                                          | 2.09±.07                                                | 1.20±.02                         | 6.83±.06                                                  |
| 298.15   | 4.25±.10                                                      | 4.25                                                          | 1.29±.09                                                | 1.13±.03                         | 4.12±.08                                                  |
| 308.15   | 3.29±.09                                                      | 3.29                                                          | .72±.06                                                 | 1.09±.01                         | 3.03±.05                                                  |
| Mb       |                                                               |                                                               |                                                         |                                  |                                                          |
| 288.15   | 9.29±.05                                                      | 9.29                                                          | 5.25±.07                                                | 1.24±.05                         | 8.99±.03                                                  |
| 298.15   | 7.24±.07                                                      | 7.24                                                          | 3.16±.06                                                | 1.20±.02                         | 7.12±.09                                                  |
| 308.15   | 4.83±.08                                                      | 4.83                                                          | 2.34±.04                                                | 1.17±.02                         | 4.27±.05                                                  |

Figure 3. Bar graph showing the effect of temperature on the binding of amsacrine with Hb and Mb.

3.5. Three-dimensional fluorescence studies
In order to further investigate ligand-induced alteration in the protein’s conformation, 3D fluorescence spectra of Hb and Mb were recorded in the absence and in the presence of increasing concentration of amsacrine. Supplementary Figure S2 shows 3D fluorescence spectra and corresponding contour maps of Hb and Mb (Supplementary Figure S2 A, B and C, D) and their complexes with amsacrine (Supplementary Figure S2 E, F and G, H) and the corresponding parameters are depicted in Table 2. Peaks a and peak b represent first-order Rayleigh scattering ($\lambda_{ex} = \lambda_{em}$) and second-order Rayleigh scattering peak ($\lambda_{ex} = 2\lambda_{em}$), respectively. Peak 1 ($\lambda_{ex} = 280$ nm, $\lambda_{em} = 332.50$ nm) is attributed to the intrinsic fluorescence spectral behavior of Trp and Tyr residues (Kang et al., 2004). It was observed that the fluorescence intensity decreased and the Stokes ($\Delta\lambda$) shift increased on binding of amsacrine, which is suggestive of the fact that the conformation of heme proteins was altered with concomitant exposure of some hydrophobic patches that remained buried before (Peng, Ding, Peng, & Sun, 2014). Peak 2 ($\lambda_{ex} = 230$ nm, $\lambda_{em} = 322.00$ nm) is due to the $n \rightarrow \pi^*$ transition of the polypeptide backbone. In the presence of amsacrine the fluorescence intensity of both peaks diminished, but to different extents suggesting significant conformational changes in the heme proteins upon interaction with amsacrine.

3.6. CD spectroscopy
CD spectroscopy is a valuable technique for detecting conformational changes in proteins as the spectra are sensitive to small alterations in the protein structure. The far ultraviolet CD spectra of Hb and Mb contained two negative minima at 208 and 222 nm. This is characteristic of the $\alpha$-helical structure (Woody, 1995). The 208 nm band corresponds to $\pi^* \rightarrow \pi^*$ transition of the $\alpha$-helix and the 222 nm band is due to $n \rightarrow \pi^*$ transition for both the $\alpha$-helix and unordered coil. The CD spectra of Hb and Mb in the presence of various concentration of amsacrine are presented in Figure 5. The intensity of the 208 and 222 nm bands decreased with the addition of amsacrine without causing any significant shift of the peak. This suggests loss of $\alpha$-helical secondary structure content upon interaction with amsacrine. The $\alpha$-helical content of Hb and Mb was calculated to be 42 and 80%, respectively, as described previously (Chen, Yang, & Martinez,
The helical content was reduced by 23 and 8%, respectively, for Hb and Mb, upon binding with amsacrine. The decrease in the α-helical content may suggest disruption of the H-bond network leading to an unfolding of the polypeptide chains of Hb. The interaction also leads to decrease in the hydrophobicity of the microenvironment of the chromophore. The conformational change due to amsacrine binding is much less pronounced in Mb compared to Hb, implying that the secondary structure of Hb is more perturbed than Mb due to binding. No significant change was observed for the CD spectra in the near UV region.

Both Hb and Mb exhibited characteristic CD spectra in the Soret band region with a large positive maxima at 410 and 407 nm, respectively. The intensity of the positive band maxima decreased in both cases with no concomitant shift of the maxima in the presence of amsacrine and change was more in Hb than Mb for 410 and 407 nm peaks, respectively, revealing a structural change occurring in the heme part upon amsacrine binding.

### 3.7. FT-IR studies

Fourier transform infrared spectroscopy (FT-IR) is also very useful for secondary structure determination of proteins. The infrared spectra of heme proteins are essentially the amide-I (~1600–1700 cm⁻¹) and amide-II (~1500–1600 cm⁻¹) bands. The amide-I band resulted from carbonyl stretching vibrations of the peptide...
backbone that depends on the strength of the hydrogen bond and interactions between the amide units. Amide-II band is due to a combination of N–H in-plane bending and C–N stretching vibrations of the peptide groups (Mahato et al., 2010). The amide I band shifted from 1652 cm\(^{-1}\) in native Hb to 1648 cm\(^{-1}\) for amsacrine bound Hb and 1643 cm\(^{-1}\) in native Mb to 1640 cm\(^{-1}\) for amsacrine–Mb complex (Figure 6). The amide II band at 1545 cm\(^{-1}\) in Hb and 1544 cm\(^{-1}\) in Mb also shifted to 1546 and 1523 cm\(^{-1}\), respectively, upon complexation with amsacrine. Shift in the peak positions suggested the occurrence of changes in the secondary structure of the heme proteins.

3.8. Differential scanning calorimetry and optical thermal melting studies

In order to investigate the temperature-dependant transition in these proteins, thermal melting and differential scanning calorimetry (DSC) studies were done. Melting profile of heme proteins alone and in the presence of amsacrine was found to have a single transition phenomenon in DSC (Figure 7). Melting temperature of Hb and Mb were found to be 61 and 83 °C, respectively. The ratio between the calorimetric enthalpy (\(\Delta H_{\text{cal}}\)) and the van’t Hoff enthalpy (\(\Delta H_{\text{v}}\)) obtained for the thermal unfolding was not unity, indicating that the melting is not exhibiting a simple two-state unfolding behavior. Upon binding of amsacrine, the melting temperature was shifted to 58 and 78 °C, respectively, for Mb and Hb. Similar effect was also observed from optical melting experiments (Figure 7(A) and (B)) for both proteins. Thermal destabilization of the heme proteins indicated that the binding caused structural alteration in the proteins.

3.9. Thermodynamics of the interaction

Thermodynamic characterization of the binding was achieved by ITC experiments. Detailed calorimetric studies on the interaction of amsacrine with the Mb and Hb were performed. Figure 8 presents the representative primary data from the isothermal calorimetric titration of amsacrine to the proteins. The thermograms in both cases revealed negative peaks in the plot of power against time. This suggests exothermic binding reaction. Correction was applied by subtracting the corresponding dilution heats derived from the injection of amsacrine into the buffer alone. The resulting corrected heats were plotted as a function of mole ratio of amsacrine/protein. The data points in the lower panel of Figure 8 present

Figure 5. Circular dichroism (far UV CD) spectral changes of (A) Hb (1 μM) and (B) Mb (1 μM) in the presence of increasing concentration of amsacrine. Soret band CD spectral changes of (C) Hb (5 μM) and (D) Mb (5 μM) on interaction with amsacrine.
the experimental injection heats, and the solid lines represent the calculated best fits to the experimental data based on the “one set of sites” model. The binding affinity ($K_b$) values were $(1.21 \times 10^5)$ and $(3.59 \times 10^5)\text{M}^{-1}$, respectively, (Table 3) for the complexation to Hb and Mb, respectively. It may be recalled that a higher binding affinity value of the amsacrine binding to Mb over Hb was observed from spectroscopic experiments also. A negative value of $\Delta H^o$ demonstrated the existence of electrostatic interactions as well as hydrogen-bonding interactions (Ross & Subramanian, 1981).

ITC experiments were performed at three different temperatures, viz. 283.15, 293.15, and 303.15 K. The thermodynamic quantities deduced at these temperatures are depicted in Table 3. It can be seen that as the temperature was raised, the equilibrium binding constant decreased, the standard molar enthalpy contributions became more negative and the standard molar entropy contributions decreased (Table 3).

The constant-pressure heat capacity change ($\Delta C_p$) of amsacrine–protein complexation was determined by employing the standard relationship.
Heat capacity change data can lend valuable information about the type and magnitude of forces governing the complexation phenomenon. The observed enthalpy values varied linearly in the temperature range (283.15−303.15 K) studied, indicating that there is no measurable shift of the pre-existing equilibrium between the conformational states of the protein in the temperature span studied. The values of $\Delta C_p$ for Hb and Mb were $-135.2$, $-191.4$ cal mol$^{-1}$K$^{-1}$, respectively. There are two heat capacity contributions, from hydration and protein–protein interactions; which dominates in folding and binding depending upon various factors. The negative sign of heat capacity for globular proteins compared with other larger proteins may be attributed to the dominant role of polar group hydration upon unfolding with a small contribution from the protein chain (Richardson & Makhatadze, 2004). The nonzero and the negative values of $\Delta C_p$ in both the cases indicated that the binding is

$$\Delta C_p = \frac{\partial(\Delta H^\circ)}{\partial T}$$  \hspace{1cm} (3)
specific and accompanied by the burial of non-polar surface area (Loladze, Ermolenko, & Makhatadze, 2001). Moreover, the difference in the values between Hb and Mb indicates the different extent of hydrophobic interaction in these systems.

3.10. Effect of divalent metal ions

Trace metal ions are widely distributed in plasma and are vital to human body. Therefore, the effect of some common metal ions Mg$^{2+}$, Ca$^{2+}$, Co$^{2+}$, Cu$^{2+}$, and Zn$^{2+}$ on the binding constants of amsacrine and heme proteins were investigated by recording the fluorescence intensity in the range of 300–500 nm upon excitation at 295 nm and the results obtained are depicted in Table 4. The results indicate that Mg$^{2+}$, Ca$^{2+}$, Co$^{2+}$, Cu$^{2+}$, and Zn$^{2+}$ decreased the binding affinity of amsacrine to Hb and Mb which may be due to the competition between metal ions with amsacrine for the binding sites on the proteins.

3.11. Energy transfer from the heme protein to amsacrine and calculation of binding distance

Förster Resonance Energy Transfer (FRET) relies on the distance-dependent transfer of energy from a donor molecule to an acceptor molecule at the cost of the emission from the former molecular system (Lakowicz, 1999; Sytnik & Litvinyuk, 1996). In the protein environment, the proximity of the ligand molecule to the Trp moiety of the protein can be determined through FRET studies. According to Förster’s theory, the efficiency of FRET depends principally on the following factors: (i) the extent of overlap between the donor emission and the acceptor absorption, (ii) the orientations of the transition dipoles of the donor and the acceptor, and (iii) the distance between the donor and the acceptor. If the emission spectrum of the donor significantly overlaps with the absorption spectrum of the acceptor, the donor–acceptor pairs are considered to be within Förster distance, and the possibility of energy transfer as described above can be envisaged. The overlap (crossed) of the absorbance spectra of amsacrine with the fluorescence emission spectrum of Hb and Mb is shown in Figure 9(A) and (B). FRET efficiency of the donor (Trp) was estimated to calculate the distance of donor acceptor pair using the standard relationships as described previously (Hazra et al., 2013; Saha, Rakshit, & Pal, 2013). The distance between amsacrine and Trp residues in Hb and Mb were found to be 2.77 and 3.33 nm, respectively. The distance between the donor and the acceptor ($r_0$) and the critical energy transfer distance ($R_0$) are much lower than 7 nm value indicating a high probability of energy transfer from the Trp residues of the protein to the amsacrine (Valeur, 2001) as a result of formation of strong ground-state complexes (Wang et al., 2007).

4. Conclusions

Spectroscopic evidence suggests that amsacrine binds with the heme proteins through ground-state complex formation. The binding affinity was about three times

| Metal ions | $K \times 10^5$ (M$^{-1}$) | Stoichiometry ($n$) |
|------------|-----------------------------|---------------------|
| Hb         |                             |                     |
| Ca$^{2+}$  | 1.04 ± .03                  | 1.18 ± .02          |
| Mg$^{2+}$  | 1.00 ± .02                  | 1.19 ± .01          |
| Co$^{2+}$  | .83 ± .04                   | 1.20 ± .02          |
| Cu$^{2+}$  | .79 ± .02                   | 1.22 ± .03          |
| Zn$^{2+}$  | .74 ± .02                   | 1.11 ± .02          |
| Mb         |                             |                     |
| Ca$^{2+}$  | 4.94 ± .03                  | 1.15 ± .02          |
| Mg$^{2+}$  | 4.56 ± .01                  | 1.12 ± .03          |
| Co$^{2+}$  | 3.97 ± .01                  | 1.19 ± .02          |
| Cu$^{2+}$  | 3.33 ± .02                  | 1.09 ± .01          |
| Zn$^{2+}$  | 3.15 ± .02                  | 1.12 ± .03          |
higher to Mb than Hb. Synchronous fluorescence, 3D fluorescence and CD studies revealed that a stronger conformational change took place in Hb compared to Mb upon binding of amsacrine. On binding the protein conformation was altered leading to a reduction of the α-helical organization and concomitant increase in the coiled structure, indicating a small but definitive partial unfolding of Hb. A reduction of the thermal stability was also observed from melting studies. Thermodynamics of the interaction revealed exothermic binding and was also observed from melting studies. Thermodynamic unfolding of Hb. A reduction of the thermal stability coiled structure, indicating a small but de

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