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Studies of Interaction of Iron(II) Complexes with Human Serum Albumin by Spectrophotometric and Dialysis Equilibrium Methods

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In this paper, we are studying about the complex formation between a metal, macromolecule and simple organic ligand have been investigated for different reasons. It is enable to determine the presence of chelating group in the *vicinal* positions for determining enzyme activity and explaining the drug mechanism.

Key Words: Iron(II) complexes, Human serum albumin, Spectrophotometry, Dialysis.

INTRODUCTION

The ternary complex formation between a metal, macromolecule and simple organic ligand have been investigated for two reasons, firstly it enable us to determine the presence of chelating groups in the *vicinal* positions for determining enzyme activity and secondly for explaining the drug mechanism. In such direction, Lerman[1,2] proposed that planar organic compounds can bind to DNA to explain the binding mode of the mutagenic acridives to DNA. Since that pivotal work, there has been considerable interest in the study of the binding of small molecules to DNA[3-10]. In studying the interaction of small molecules with DNA, metal complexes are flexible reagents which offer the opportunity to explore the effects of the central metal atom, ligands and coordination geometry on the binding event. The metal complexes bound to DNA through the non-covalent interaction generally form an important subgroup, of which the members are cations and typically contain ligands bearing extended hydrophobic area or sufaces[11]. The examples of the category of DNA binding agents are metal complexes with polypyridines or 1,10-phenanthrolines and their derivatives[12]. Metal complexes of 1,10-phenanthroline or modified phenanthroline, are particularly alternative because they can effectively bind to DNA in different modes of interaction[13,14].

In recent years, much interest has been focused on the DNA binding of mixed ligand complexes which contain both phenanthroline or bipyridine and their modified forms[15,16]. The modified phenanthroline ligands are designed to achieve more effective binding affinity of the complexes to DNA[17,18]. In addition to the DNA binding of porphyrin and metallo-porphyrin[19,20]. Mudasir *et al.*[21,22], have recently reported the non-covalent interaction of Fe(II) mixed-ligand complexes with DNA[21,22].

The interaction of native call thymus DNA with Fe(II)-dipyrido(3,2-9,2l-3l-C) phenazine complex has been studied by variable temperature UV adsorption spectrophotometry, circular dichroism (CD) and fluorences spectroscopy[23]. The results obtained in solution at various ionic strength values give support for a tight interaction binding of the Fe(II) complexes with DNA[23]. The salt dependent binding of racemic iron(II) mixed ligand complexes containing 1,10-phenanthroline and dipyridophenazine to calf thymus DNA has been characterized by UV-vis spectrophotometric titration[24]. The equilibrium binding constant of the iron(II) complexes to DNA decreases with the salt concentration is the solution. The slope has been found to be 0.49, suggesting that in addition to interaction, considerable electrostatic interaction is also involved in the DNA binding of iron(II) complex.

However, a literature survey revealed that no such studies between Fe(II) complexes and human serum albumin (HSA) have been made. This work reports a study using dialysis equilibrium and spectrophotometric methods on the binding of Fe(II) complexes of 2,2′-bipyridyl and 1,10-phenanthroline to human serum albumin. The effect of temperature and pH on the binding has been discussed.

EXPERIMENTAL

Human serum albumin (Sigma product, mol. wt. 69000) was dissolved in double distilled water and strength of protein solution was determined by evaporating its known volume in an air oven at 100-115 ºC as well as by biuret method.

Stock solution of iron(II) phenanthroline and iron(II) bipyridyl were prepared *in situ* by addition of three equivalents of the respective ligands to one mole of iron(II) sulphate, the concentration of each complex prepared was 2×10^{-4} M. Sodium acetate-acetic acid buffer was prepared by mixing 0.2 M of each reagent (for pH 5.57) and recording the pH on a systronic pH meter. Similarly buffer of pH 7.50 was prepared by mixing of desired amounts of disodium and mono sodium hydrogen phosphates at a pH meter.

Techniques

Spectrophotometric measurements: The absorption spectra of the complexes (2×10^{-4} M) were drawn on a Elico UV-spectrophotometer. The effect of protein (0.7 %) was seen at pH 5.57 and 7.50 corresponding to carboxyl and imidazolium deprotonation. For there measurements buffer blanks were used for solution of complexes, while protein blanks were used for determining the spectra of protein metal complexes. The absorbance of protein-complexes mixtures varying to each other at respective pH-values using protein blank in all the cases.

Dialysis equilibrium measurements: This method consists of a vessel divided in two parts by a membrane usually prepared from cellophane tubings, which is permeable to water and small molecules[25]. If the macromolecule is confined to one part and if it combines with complexes cations, then at equilibrium the total ion concentration in the protein part is greater than that in the outside. The difference in the two concentrations is a measure of the ion bound to the protein. In actual practise two errors may be expected in dialysis method, (i) some asymmetry in the distribution of ions may exist because of the Dorman effect[26] and so the allowance should be made for this in calculation[27] or an inert electrolyte may be added to remove it, (ii) some adsorption of ions may occur on the membrane. This can be removed by adjusting a blank. The cellephane tubings were soaked in a solution of supporting electrolyte *i.e.*, KCl solution and then filled with 5.0 mL of protein solution. Several such sets were prepared at the desired pH by means of buffers of pH 5.57 and 7.50. These bags were inserted in 5.0 mL of metal-coordinate solution of known concentration. All the bags were placed on a shaker at 25 ºC for a period of 48 h. These experiments were preformed at 25 and 35 ºC, respectively. The bags were removed and the external solutions were analyzed on Elico-UV-spectrophotometer at 490 nm. Blanks were also run to determine the binding of complex to the bag and these were found to be negligible. The equilibrium dialysis results are shown in Tables 1-4, while the spectrophotometric results are expressed in the form of absorbance *versus* wavelength curves (Figs. 1-5).

TABLE-1
BINDING PARAMETERS OF Fe(II)-BIPYRIDYL COMPLEX TO HUMAN SERUM ALBUMIN (5 g/L FIXED) BY DIALYSIS EQUILIBRIUM METHOD AT 25 ºC, pH 5.57 AND μ = 0.15 M

Molar conc.^{n} of complex $(C_0) \times 10^5$	Free conc.^{n} of complex $(C_F) \times 10^5$ M	Bound conc.^{n} of complex $(C_B) \times 10^5$ M	V_M	$V_M/C_F \times 10^{-5}$	$1/C_F \times 10^{-5}$	$1/V_M$
4	0.28	3.72	0.516	1.842	3.571	1.937
8	0.52	7.48	1.038	1.996	1.923	0.963
12	1.20	10.80	1.388	1.156	0.833	0.720
16	2.00	14.00	1.944	0.972	0.500	0.514
20	3.76	16.24	2.255	0.599	0.265	0.443
24	3.60	20.40	2.833	0.786	0.277	0.352

TABLE-2
BINDING PARAMETERS OF Fe(II)-BIPYRIDYL COMPLEX TO HUMAN SERUM ALBUMIN (5 g/L FIXED) BY DIALYSIS EQUILIBRIUM METHOD AT 35 ºC, pH 5.57 AND μ = 0.15 M

Molar conc.^{n} of complex $(C_0) \times 10^5$	Free conc.^{n} of complex $(C_F) \times 10^5$ M	Bound conc.^{n} of complex $(C_B) \times 10^5$ M	V_M	$V_M/C_F \times 10^{-5}$	$1/C_F \times 10^{-5}$	$1/V_M$
4	0.28	3.72	0.516	1.842	3.571	1.937
8	0.52	7.48	1.038	1.996	1.923	0.963
12	1.20	10.80	1.388	1.156	0.833	0.720
16	2.00	14.00	1.944	0.972	0.500	0.514
20	3.76	16.24	2.255	0.599	0.265	0.433
24	3.60	20.40	2.833	0.786	0.277	0.352

TABLE-3
BINDING PARAMETERS OF Fe(II)-PHENANTHROLINE COMPLEX TO HUMAN SERUM ALBUMIN (5 g/L FIXED) BY DIALYSIS EQUILIBRIUM METHOD AT 25 ºC, pH 5.57 AND μ = 0.15 M

Molar conc.^{n} of complex $(C_0) \times 10^5$	Free conc.^{n} of complex $(C_F) \times 10^5$ M	Bound conc.^{n} of complex $(C_B) \times 10^5$ M	V_M	$V_M/C_F \times 10^{-5}$	$1/C_F \times 10^{-5}$	$1/V_M$
4	2.095	1.905	0.264	0.120	0.455	3.788
8	5.635	2.365	0.328	0.058	0.177	3.048
12	9.130	2.870	0.398	0.044	0.109	2.513
16	12.260	3.740	0.519	0.042	0.081	1.927
20	15.530	4.470	0.620	0.039	0.064	1.613
24	18.642	5.358	0.744	0.039	0.053	1.344

TABLE-4
BINDING PARAMETERS OF Fe(II)-PHENANTHROLINE COMPLEX TO HUMAN SERUM ALBUMIN (5 g/L FIXED) BY DIALYSIS EQUILIBRIUM METHOD AT 35 ºC, pH 5.57 AND μ = 0.15 M

Molar conc.^{n} of complex (C_0) × 10^5	Free conc.^{n} of complex (C_F) × 10^5 M	Bound conc.^{n} of complex (C_B) × 10^5 M	V_M	$V_M/C_F \times 10^{-5}$	$1/C_F \times 10^{-5}$	$1/V_M$
4	2.272	1.728	0.240	0.105	0.440	4.116
8	5.988	2.012	0.279	0.047	0.167	3.584
12	9.560	2.440	0.338	0.035	0.104	2.958
16	12.975	3.025	0.420	0.032	0.770	2.380
20	16.230	3.770	0.524	0.032	0.610	1.908
24	19.140	4.860	0.675	0.035	0.052	1.481

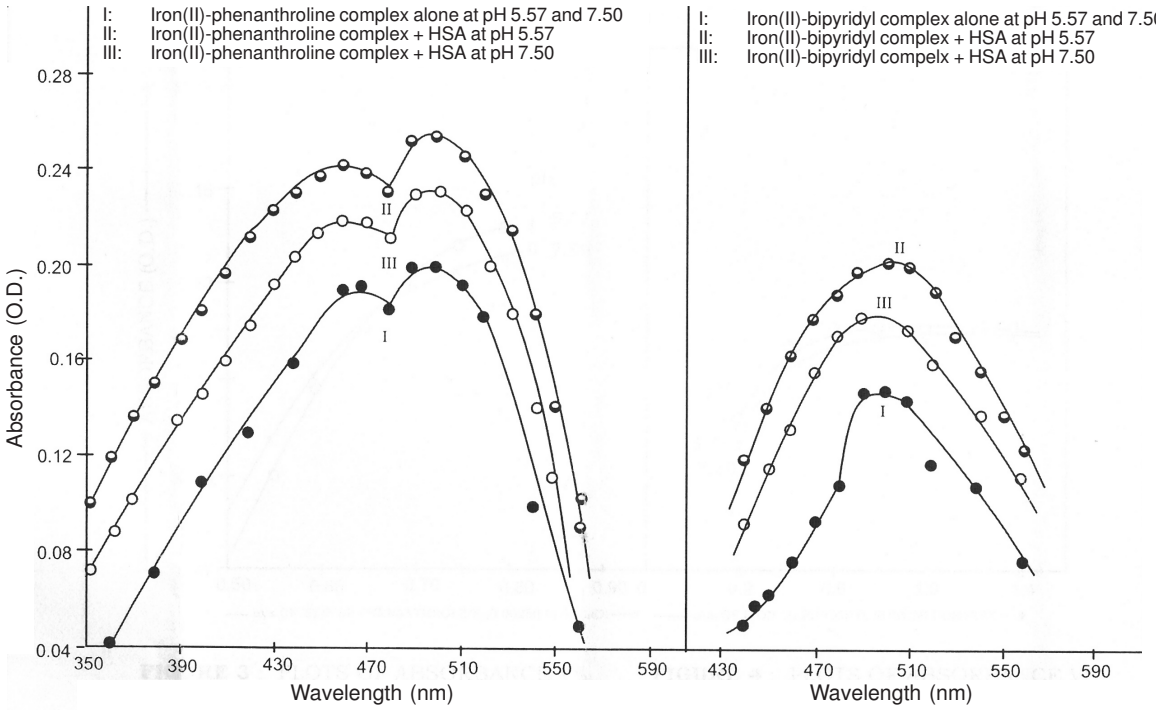

Fig. 1. Spectrum of iron(II)-phenanthroline complex in absence and presence of human serum albumin

Fig. 2. Spectrum of iron(II)-bipyridle complex in absence and presence of human serum albumin

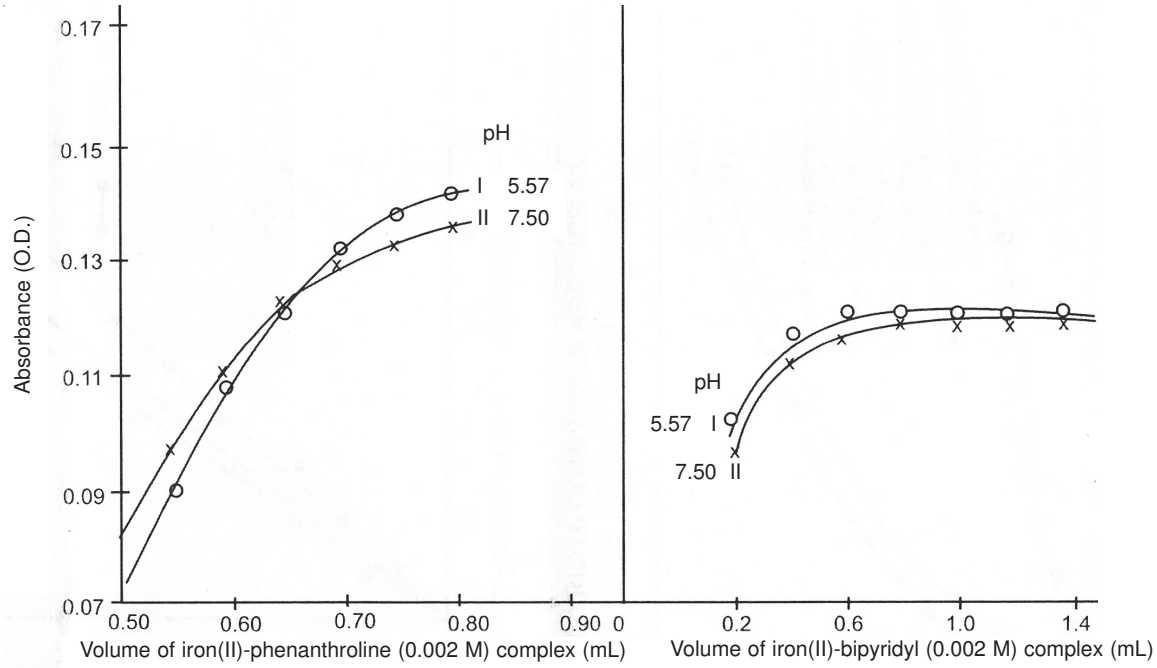

Fig. 3. Plots of absorbance *versus* volume in mL of iron(II)-phenanthroline complex in presence of 0.2 % human serum albumin (total volume 10 mL)

Fig. 4. Plots of absorbance *versus* volume in mL of iron(II)-bipyridyl complex in presence of 0.2 % human serum albumin (total volume 10 mL)

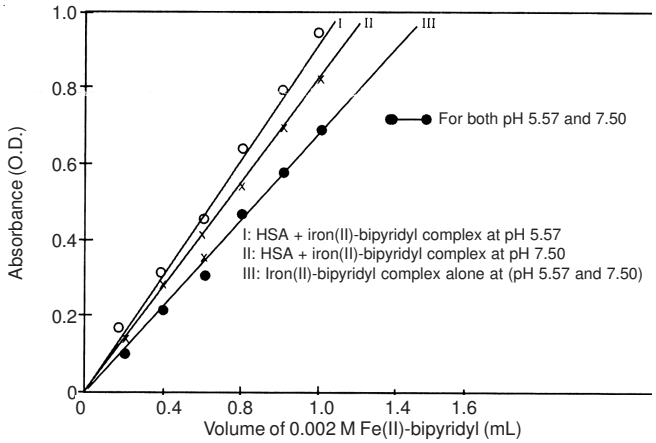

Fig. 5. Plots of absorbances *versus* volume in mL of 0.002 M iron(II)-bipyridyl complex in absence and presence of 0.7 % human serum albumin (total volume 10 mL)

RESULTS AND DISCUSSION

The absorption spectra of Fe(II)phenanthroline and F(II)-bipyridyl complexes are shown in Figs. 1 and 2. Fe(II)-phenanthroline complex revealed anomalous spectrum as it exhibited a shoulder at 470 nm and a broad maximum between 490-510 nm, whereas Fe(II)-bipyridyl showed in sharp maximum at a wavelength of 490. The addition of human serum albumin in each case did not altered the absorption maxima, but the absorbance was greatly increased. There is no change in the general architect of the spectrum of the respective iron complex by the added human serum albumin. The increase in the absorbance of the complexes by the added human serum albumin is a sign of the deepening of the colour intensity and therefore provided a qualitative evidence of the binding of cationic complexes with protein.

Iron(II)-phenanthroline complexes exhibited an absorbance of 0.220 at 490 nm, while in presence of human serum albumin, it was found to be 0.262 and 0.248 at pH values 5.57 and 7.50, respectively. The net difference of 0.042 and 0.028 in absorbance is an indication of the fixation of the complex ion on reactive sites of human serum albumin. In case if Fe(II)-bipyridyl complex ion the absorbance at maxima is 0.120, but in the presence of macromolecule it attained a value of 0.220 and 0.190 at pH 5.57, respectively. The net difference is equal to 0.100 and 0.070 at two pH levels. The larger value of absorbance difference in case of Fe(II)-bipyridyl than Fe(II)-penanthroline complex ion indicates more binding of former than latter with the reactive groups of protein. These findings go to show that human serum albumin produces more deepening of colour of Fe(II)-bipyridyl than that of Fe(II)-phenanthroline complex. The Fe(II)-bipyridyl is more reactive than Fe(II)-penanthroline is occupying the reactive sites of human serum albumin.

The spectrophotometric titration carried at pH 5.57 and 7.50 are shown in Figs. 3-5. The figures although revelaed difference in absorbance, but the inflexions are seen at the same concentration of complex or protein. These observation go to show that binding is not affected by a change in pH from 5.57-7.50, since the imidazolium groups can not coordinate with iron complexes as coordination number of Fe(II) is fully saturated in both the complexes. The spectrophotometric observations indicated the involvement of carboxyl groups only in the binding process.

The quantitative observations of binding of Fe(II) complexes with human serum albumin have been studied at pH 5.57 and two different temperatures, *viz*., 25 and 35 ºC by dialysis equilibrium method. In these studies, two temperatures were used to find the thermodynamic parameters and such parameters are used in order to determine the energetic bonding. The data obtained for Fe(II)-bipyridyl and Fe(II)-phenanthroline are given in Tables 1-5 and graphically shown in Fig. 6 in terms of V_M, the number of moles of metal coordinate bound per mole of human serum albumin and C_F, the concentration of free equilibrium metal coordinate in the solution with the protein.

TABLE-5
BINDING CONSTANTS OF $[Fe(II)-(Phen)_3]^{2+}$ AND $[Fe(II)-(bipyridyl)_3]^{2+}$ TO HUMAN SERUM ALBUMIN (HSA) BY DIALYSIS EQUILIBRIUM METHOD AT pH 5.57 AND μ = 0.15 M

Constant	$[Fe(II)(Phen)_3]^{2+}$-HSA system		$[Fe(II)(bipyridyl)_3]^{2+}$-HSA system	
	25 ºC	35 ºC	25 ºC	35 ºC
n	1.500	1.500	2.700	2.300
log K	1.994	1.994	3.594	3.554
ΔG° kcal/mol	-2.709	2.800	-4.883	-4.991
ΔH° kcal/mol	–	–	-1.674	–
ΔS° kcal/mol	–	–	+10.768	+10.769

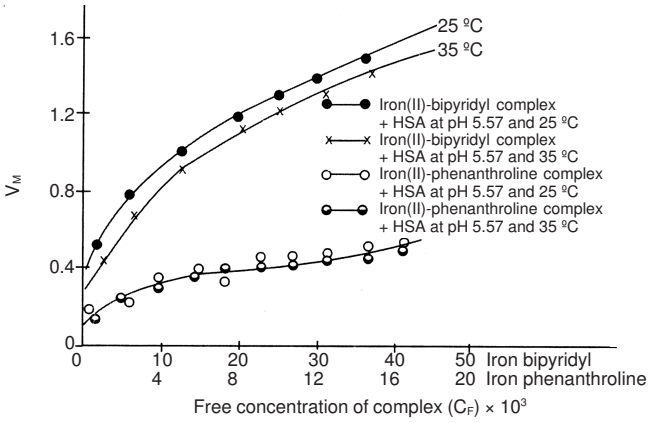

Fig. 6. Plots of V_M *versus* free concentration of complex (C_F) for iron(II)complexes-human serum albumin system

The results showed that the Fe(II)-bipyridyl complexes is bound more strongly to human serum albumin that of phenanthroline complex. In these two cases, the number of moles of metal coordinates bound are much less than the total carboxyl groups of human serum albumin molecule *i.e.*, 98[28]. A slight elevated value of V_M at pH 7.50 has been attributed only to exposes of more carboxyl for binding with the complex cations. All possibilities of coordination either with water molecules or with the nitrogen donor atom of protein are avoided owing to the fully saturation of auxiliar valency of the central metal atom inside the atmosphere on the complex. It is apparent that only few carboxyl groups of the preotein are involved in the interaction. It may be inferred that either the size of the these metal coordinates or the location of the positive charge on them in such that the carboxylate anion on

protein could not offer opportunity to be attracted by the metal coordinate cations. It may also be that majority of carboxyl are sterically hindered by the neighbouring amino acid residues, hence they are not easily accessible to interact with cationic coordinate at pH 5.57. Although human serum albumin cantains the N-terminal sequence Asp-Ala-His and is able to strongly bind the metal ions with a high affinity for nitrogen through the donor set (NH_2, N^-, N^{2-}, Nimid) but such condition is not possible at pH 5.57. Thus all other possibilities of coordination either with water molecule or with donor nitrogen atoms protein are neglected due to the complete saturation of auxillary valence of the central iron atom inside the coordinate sphere of the coordinate. The system in hand is similar to that reported by Klotz *et al.*[29] where a ternary complex formation between dye, metal and BSA has been suggested.

Inspite of the presence of same amount of positive charge, the two Fe(II) complexes revealed marked differences in their binding pattern. One of the main reason could be the ion-pair forming tendency of Fe(II)-phenanthroline complex[30], hence it might be acting as equivalent to an ion of monovalent charge, while such ion pair forming ability may be negligible in case of Fe(II)-bipyridyl complex. In order to make a quantitative comparison of the two systems it is essential to determine the binding of one metal-coordinate cation to one molecule of protein.

$$HSA\text{-}COOH \quad HSA\text{-}COO^- + H^+$$

$$[Fe(II)(Phen)_3]^{2+} + HSA\text{-}COO^- \quad [Fe(II)(Phen)_3]^{2+}\cdot HSA\text{-}COO^-$$

The equilibrium constant (K_1) is then given by the following relation:

$$K_1 = \frac{[Fe(II)(Phen)_3^{2+}\cdot HSA - COO^-]}{[Fe(II)(Phen)_3^{2+}]\cdot[HSA - COO^-]}$$

The value of equilibrium constant (K_1), K can be obtained by extra plotting a plot of V_M/C_F *versus* C_F at $C_F = 0$. At this limiting value of C_F.

$$\text{Limit}\left(\frac{V_M}{C_F}\right) = nKC_F = K_1$$

where n = number of equivalent sites on human serum albumin molecule and KC_f = intrinsic association constant for the binding. The value of K may also be determined easily by the following equation of Scatchard[31].

$$K = \frac{V_M}{[n - V_H^+ - V_M]C_F}$$

The value of n and V_{H^+} are taken from the literature of human serum albumin[20]. The values of K were found to decrease with increasing value of C_F indicated that the first binding site if stronger than the subsequent binding sites. The binding constants are compiled in Table-5.

The values of V_M/C_F (Tables 1-4) were found approximately indentical at all the free equilibrium metal coordinate concentrations (C_F). The number of binding sites (n) at 25 ºC were found to be 1.5 and 2.7 for Fe(II)-phenanthroline and Fe(II)-bipyridyl, respectively, whereas, at 35 ºC for Fe(II)-bipyridyl binding sites (n) was found to be 2.3 only. With such a limited occupancy of the binding sites it would be expected that binding at various sites would be independent of one another and V_M/C_F would have nearly same value equal to nKC_F. In such case a binding site may consist one carboxyl group if there is ion-pair formation between the cationic coordinate and the chloride ion from supporting electrocyte KCl or two carboxyl groups, if there was no ion-pair formation. The logarithm of intrinsic association constant log K for the two Fe(II) complexes are of the same order, therefore the magnitude of linking of human serum albumin in case of both complexes is the same and V_M/C_F would have nearly same value equal to nKC_F. In such case a binding site may consist one carboxyl group if there is ion-pair formation between the cationic coordinate and the chloride ion from supporting electrolyte KCl or two carboxyl groups, if there was no ions-pair formation. The logarithm of intrinsic association constant log K for the two Fe(II) complexes are of the same order, therefore the magnitude of linking of human serum albumin in case of both complexes is the same.

The interaction of Fe(II)-complexes with human serum albumin was found to be temperature independent in case of Fe(II)-phenanthroline complex, but the free energy change (ΔG^o) at 25 ºC suggested binding with one class of sites. In the case of Fe(II)-bipyridyl complex binding was found to be slight dependent on temperature. The enthalpy change (ΔH^o) for Fe(II)-bipyridyl-human serum albumin was found to be -1.674 kcal/mol, while the entropy change (ΔS^o) were found positive *i.e.*, +10.768 and 10.769 cal/degree/mol, at 25 and 35 ºC, respectively. The positive sign of entropy change (ΔS^o) signifies of few human serum albumin bound and transfer of the small molecule to the reactive site on the human serum albumin molecule. The negative sign of enthalpy change (ΔH^o) indicated the electrostatic attraction between metal coordinate cation and anionic sites of the human serum albumin[32-35].

According to views of Ross and Subramanian[36], the positive ΔH^o and ΔS^o values are associated with hydrophobic interaction. The negative ΔH and ΔS values are associated with hydrogen bonding and van der Waals interaction is low dielectric medium. Finally low positive or negative ΔH^o and positive ΔS^o values are characterized by electrostatic interactions. From Table-5, it can be seen that the negative sign for ΔG^o indicated the spontanesly of the binding of Fe(II) complex with human serum albumin. The enthalpy change of binding is consistant with the small heat of binding with Cu(II) complex with DNA[37].

However, the higher value of logarithm of association constant (log K) and binding sites (n) Fe(II)-bipyridyl than Fe(II)-phenanthroline suggested stronger consideration for the former than that of latter.

REFERENCES

1. L.S. Lerman, *J. Mol. Biol.*, **3**, 18 (1961).
2. L.S. Lerman, *Proc. Nat. Acad. Sci. USA*, **49**, 94 (1963).
3. S. Mahadevan and M. Palaniandavar, *Inorg. Chem.*, **37**, 3927 (1998).
4. C.M. Dupureur and J.K. Barton, *Inorg. Chem.*, **36**, 33 (1997).
5. R.E. Holmin, E.D.A. Stemp and J.K. Barton, *Inorg. Chem.*, **37**, 29 (1998).
6. D.R. McMillin and K.M. McNett, *Chem. Rev.*, **98**, 1201 (1998).
7. S.D. Choi, M.S. Kim, S.K. Kim, P. Lincoln, E. Tuite and B. Norden, *Biochemistry*, **36**, 214 (1997).
8. E. Tuite, P. Lincoln and B. Norden, *J. Am. Chem. Soc.*, **119**, 239 (1997).

9. J.G. Collins, A.D. Sleeman, J.R. Alrich-Wright, I. Gerguric and T.W. Hambley, *Inorg. Chem.*, **37**, 3133 (1998).
10. C. Moucheron, A.K. Mesmaeker and S. Choua, *Inorg. Chem.*, **36**, 584 (1997).
11. R. Fiel, *J. Biomol. Struct. Dyn.*, **6**, 1259 (1989).
12. R.M. Hartshorn and J.K. Barton, *J. Am. Chem. Soc.*, **114**, 5919 (1992).
13. C.S. Chow and F.M. Bogdan, *Chem. Rev.*, **97**, 1989 (1997).
14. K. Gisselfalt, P. Lincoln, B. Nordin and M. Jonsson, *J. Phys. Chem.*, **B104**, 3651 (2000).
15. Mudasir, N. Yoshioka and H. Inoue, *Transition Met. Chem.*, **24**, 210 (1999)..
16. I. Hag, P. Lincoln, D. Suh, B. Norden, B.Z. Chowdhry, J.B. Chaires, *J. Am. Chem. Soc.*, **117**, 4788 (1995).
17. A. Ambroise and B.G. Maiya, *Inorg. Chem.*, **39**, 4256 (2000).
18. A. Ambroise and B.G. Maiya, *Inorg. Chem.*, **39**, 4264 (2000).
19. D.H. Tjahjona, T. Akutsu, N. Yoshioka and H. Inoue, *Biochem. Biol. Phys. Acta*, **1472**, 333 (1999).
20. D.H. Tjahjona, T. Yamanoto, S. Ichimoto, N. Yoshioka and H. Inoue, *J. Chem. Soc. Perkin Trans. I*, 3077 (2000).
21. Mudasir, K. Wijaya, N. Yoshioka and H. Inoue, *J. Inorg. Biochem*., **94**, 263 (2003).
22. Mudasir, K. Wijaya, E.T. Wahyuni, N. Yoshioka and H. Inoue, *Biophys. Chem.*, **121**, 44 (2006).
23. A. Terenzi, G. Barone, A. Silvestri, A.M. Giuliani, A. Ruggivello and V.T. Liveri, *J. Inorg. Biochem.*, **103**, 1 (2009).
24. B. Onfelt, P. Lincoln and B. Norden, *J. Am. Chem. Soc.*, **121**, 10846 (1999).
25. I.R. Hughes and I.M. Klotz, In ed.: D. Glick, Method of Biochemical Analysis, Interscience Publications, Inc. New York, Vol. 3, p. 265 (1956).
26. G.F. Danan, *Chem. Rev.*, **1**, 73 (1924).
27. S. Belman, *J. Am. Chem. Soc.*, **86**, 2154 (1963).
28. K. Murayama and B.C. Matuscwicz, *J. Phys. Chem.,* **105B**, 4763 (2001).
29. I.M. Klotz and W.S. Lohming, *J. Am. Chem. Soc.*, **77**, 6414 (1955).
30. J.E. Dicknes, F. Basolo and H.M. Neumann, *J. Am. Chem. Soc.*, **79**, 1286 (1957).
31. G. Scatchard, *Ann. N.Y. Acad. Sci*., **51**, 660 (1949).
32. J.P.S. Arora, R.P. Singh, D. Soam and R. Sharma, *Bioelectrochem. Bioenerg.*, **10**, 57 (1983).
33. J.P.S. Arora, R.P. Singh, D. Soam, S.P. Singh and R. Kumar, *Bioelectrochem. Bioenerg.*, **10**, 441 (1983).
34. J.P.S. Arora, R.P. Singh,D. Soam, S.P. Singh and R. Sharma, *Bioelectrochem. Bioenerg.*, **10**, 289 (1983).
35. C.B. Murphy, Y. Zhang, T. Troxler, V. Ferry, J.J. Mertin and W.E. Jones, *J. Phys. Chem.*, **108B**, 1537 (2004).
36. P.D. Ross and S. Subramaniam, *Biochemistry*, **20**, 3096 (1981).
37. S.S. Zhange, S.Y. Niu, G.F. Jie, Y. Li, K. Kiao and F. Yang, *J. Ind. Chem. Soc.*, **82**, 393 (2005).