# DNA Binding of Iron(II)-Phenanthroline Complexes: Effect of Methyl Substitution on Thermodynamic Parameters

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The influence of methyl substitution on the thermodynamic parameters for the binding of $[Fe(DMP)_3]^{2+}$ and $[Fe(TMP)_3]^{2+}$ (DMP = 4,7-dimethyl-1,10-phenanthroline, TMP = 3,4,7,8-tetramethyl-1,10-phenanthroline) to calf thymus DNA (ct-DNA) has been studied by determining their equilibrium binding constants ($K_b$) at various salt concentrations and temperatures. $K_b$ of the iron(II) complexes to ct-DNA decreases with the salt concentration in the solution, suggesting considerable electrostatic interaction in the ct-DNA binding of the iron(II) complexes. In contrast, $K_b$ of the DNA binding increases with temperature, indicating that the DNA binding reaction of the complex is endothermic and entropically driven. The evaluation of the non-electrostatic binding constant ($K^0{}_t$) based on polyelectrolyte theory has revealed that the $K^0{}_t$ portions of the total binding constant ($K_b$) are relatively large and reach 46.4 % for $[Fe(DMP)_3]^{2+}$ at $[Na^+]$ = 0.075 M and 43.9 % for $[Fe(TMP)_3]^{2+}$ at $[Na^+]$ = 0.100 M. The contribution of non-electrostatic binding free energy ($\Delta G^0_t$) to total binding free energy change ($\Delta G^0$) is extremely large, *i. e.* > 90 % for both iron(II) complexes at $[Na^+]$ = 0.05 M, suggesting that the stabilization of the DNA binding is mainly contributed from the non-electrostatic process. The effect of methyl substitution on electrostatic ($\Delta G^0_{pe}$) and non-electrostatic ($\Delta G^0_t$) binding free energy changes has been systematically evaluated using the quantity of $\Delta\Delta G^0_{pe}$ and $\Delta\Delta G^0_t$ relative to that of the parent iron(II) complex, $[Fe(phen)_3]^{2+}$. The results indicate that the substitution of hydrogen atoms in the phen ligand by methyl groups decreases slightly the electrostatic binding free energy changes, but tremendously increases the non-electrostatic ones to yield net binding free energy changes which are more favorable for the ct-DNA binding.

*Key words:* DNA-binding Ligand, 1,10-Phenanthroline, Iron(II), Thermodynamic Parameters

## Introduction

Over the past two decades, there has been considerable interest in the studies of the interaction of DNA with transition metal complexes containing planar polycyclic heteroaromatic ligands [1 – 5]. These studies provide routes towards a rational development of chemotherapeutic agents [6], sensitive chemical probes of DNA structure in the solution [7], as well as tools for molecular biologists to dissect genetic expression [8]. In general, transition metal complexes containing polypyridyl ligands such as 1,10-phenanthroline (phen) and its modified versions can bind to DNA in non-covalent interactions such as electrostatic binding, groove binding, and intercalative or partial intercalative binding [1, 2]. Many metal complexes have been synthesized using various phen modified ligands with the purpose of enhancing their interaction with DNA [9, 10], and their DNA photocleavage activity has also been investigated [11 – 17]. However, most of the studies have mainly focused on metal complexes containing fully planar ligands, while metal complexes containing substituted ligands such as methyl groups have been rarely reported. In fact some of these complexes also exhibit very interesting properties upon binding to DNA [18 – 20]. A variation in the nature and position of the substituents at the binding site of the ligand can create some interesting differences in the space configuration and electron density distribution of the metal complexes, resulting in differences in spectral profiles, DNA-binding properties, enantioselectivities, and even DNA photocleavage activities [12]. Studies

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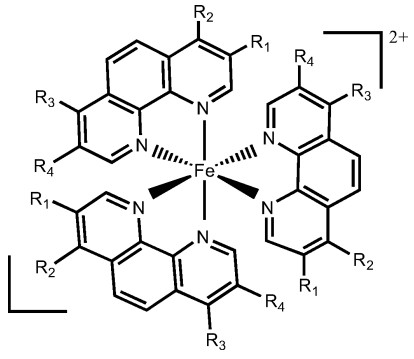


$R_1 = R_2 = R_3 = R_4 = H$: phen
$R_1 = R_4 = H$; $R_2 = R_3 = CH_3$: DMP
$R_1 = R_2 = R_3 = R_4 = CH_3$: TMP

Fig. 1. Chemical structure of iron(II)-phenanthroline complexes used for studies. Only the $\Lambda$-enantiomers are shown.

of such differences can be very useful to thoroughly understand the binding mechanism of metal complexes to DNA. Furthermore, studies on the DNA binding of metal complexes have been exhaustively dominated by elucidating their binding mode, sequence selectivity and the possible structure of DNA-metal complexes. Apart from the determination of the binding constants, the thermodynamic parameters of their DNA binding, which are essential for a better understanding of the factors that drive the binding event, have not been studied in any detail. We have recently investigated the effect of phenyl substituents and phenazine moieties on the thermodynamic parameters of a series of iron(II) mixed-ligand complexes containing phen and dip (4,7-diphenyl-phen) or dppz (dipyrido[2,3-*a*:2′,3′-*c*]phenazine) upon binding to DNA [21, 22]. On the basis of polyelectrolyte theory, we have also dissected the equilibrium binding constant ($K_b$) and binding free energy change ($\Delta G^0$) into their electrostatic and non-electrostatic portions. The last two parameters have further been analyzed and used successfully to evaluate the effect of charge and type of substituents of the DNA binding ligand on the stabilization of DNA binding events [22].

In the present study, by using a similar method, we have investigated the effect of methyl substitution at the 3, 4, 7 and 8 position of the phen ligand in $[Fe(DMP)_3]^{2+}$ (DMP = 4,7-dimethyl-phen) and $[Fe(TMP)_3]^{2+}$ (TMP = 3,4,7,8-tetramethyl-phen) complexes (Fig. 1) on the thermodynamic parameters of their DNA binding. In addition, enthalpy ($\Delta H^0$) and entropy ($\Delta S^0$) changes have been obtained from the van't Hoff plot by determining $K_b$ at various temperatures, and electrostatic, and non-electrostatic contributions of $K_b$ and $\Delta G^0$ have been evaluated by polyelectrolyte theory after determining $K_b$ at various concentrations of salt (NaCl). The data obtained have been further analyzed and compared to those previously reported to get some insight into the effect of methyl substitution on the stabilization energy of the DNA binding.

## Experimental Section

*Chemicals*

1,10-Phenanthroline (phen), 3,4,7,8-tetramethyl-1,10-phenanthroline (TMP) and 4,7-dimethyl-1,10-phenanthroline (DMP) were obtained from Wako Pure Chemicals, Ltd. (Japan), Aldrich Chem. Co. and Tokyo Kasei Chemical Co., respectively. Iron(II) complexes of phen and its derivatives were prepared as their perchlorate salts. Tris(phen)iron(II) perchlorate, $[Fe(phen)_3](ClO_4)_2$, was synthesized according to the literature procedure [23]. Tris(DMP)iron(II) perchlorate, $[Fe(DMP)_3](ClO_4)_2$, and tris(TMP)iron(II) perchlorate, $[Fe(TMP)_3](ClO_4)_2$, were prepared by a modification of the literature procedures [24] as reported previously [25]. Both procedures in general consisted of dissolving Mohr's salt, $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, and the ligands in a mole ratio of at least 1 : 3 in hot water (*ca.* 80 °C) containing 10 – 50 % of ethanol and 1 % of ascorbic acid, followed by stepwise addition of sodium perchlorate monohydrate. The crystalline iron(II) complexes were washed with water, recrystallized from hot water and dried *in vacuo*. The products were identified by elemental analysis and UV/vis absorption spectroscopy. The concentration of iron(II) complexes for DNA binding studies was calculated spectrophotometrically using the molar absorptivity ($\varepsilon$) of 11900 $M^{-1}\,cm^{-1}$ (510 nm), 13800 $M^{-1}\,cm^{-1}$ (512 nm) and 17800 $M^{-1}\,cm^{-1}$ (503 nm) for $[Fe(phen)_3]^{2+}$ [26], $[Fe(DMP)_3]^{2+}$ and $[Fe(TMP)_3]^{2+}$, respectively [25]. Tris(2-amino-2-hydroxymethyl-1,3-propandiol) (Tris buffer) was purchased from Junsei Chemical Co. Ltd. (Tokyo, Japan), and sodium chloride (NaCl) for adjusting ionic strength was obtained from Wako Pure Chemical Industries (Japan). All chemicals and solvents were of analytical grade or higher and used without further purification.

*DNA sample*

Calf thymus DNA (ct-DNA) was obtained from Sigma Chemicals Co. (USA) and used as received. The solid sodium salt of DNA samples was stored below 4 °C. A stock solution of ct-DNA was prepared and stored in 5 mM Tris-HCl buffer at pH 7.2. The concentration of ct-DNA solutions was determined spectrophotometrically using the reported molar absorptivity of $\varepsilon_{259\,nm} = 1.31 \times 10^4\ M^{-1}\,cm^{-1}$ [27], and

the results were expressed in terms of base-pairs per cubic decimeter. A solution of ct-DNA (*ca.* $10^{-5}$ M in base pair, bp) in Tris-HCl buffer gave a ratio of UV absorbance at 260 and 280 nm, $A_{260}/A_{280} \geq 1.9$, indicating that the ct-DNA was sufficiently free from protein.

*Measurements of the binding constant at various salt concentrations and temperatures*

The equilibrium binding constant ($K_b$) of iron(II) complexes to ct-DNA was determined by spectrophotometric titration over the concentration range 0.005 to 0.100 M of NaCl and in the temperature range of 11 – 32 °C. A fixed amount of iron(II) complex in 5 mM Tris-HCl buffer at pH 7.2 and NaCl solution at various concentrations was titrated at a fixed temperature with increasing amounts of ct-DNA stock solutions ($10^{-6} - 10^{-4}$ M) and the hypochromicity in the corresponding MLCT (metal-to-ligand charge transfer) bands of each iron(II) complex due to metal complex-DNA interaction was monitored by a Jasco V-570 UV/vis spectrophotometer equipped with a Jasco ETC-505T cell-temperature controller and a cell magnetic stirrer. For the determination of $K_b$ at various salt concentrations, the cell compartments were thermostated at 25 ± 0.1 °C, while for those at other temperatures, the NaCl concentration was kept constant at 50 mM. The $K_b$ values were calculated by means of Eq. 1 [28]:

$$[\mathrm{DNA}]/(|\varepsilon_A - \varepsilon_F|) = [\mathrm{DNA}]/(|\varepsilon_B - \varepsilon_F|) + 1/\{K_b(|\varepsilon_B - \varepsilon_F|),\} \quad (1)$$

where $\varepsilon_A$, $\varepsilon_F$ and $\varepsilon_B$ correspond to $A_{obsd}$/[complex], the molar absorptivity for the free iron(II) complex, and the molar absorptivity of the iron(II) complex in the fully bound form, respectively. In plots of [DNA]/($|\varepsilon_A - \varepsilon_F|$) *versus* [DNA], $K_b$ is given by the ratio of the slope to the intercept. The salt concentration dependence of $K_b$ for the DNA binding of the iron(II) complexes was then evaluated by plotting ln $K_b$ *versus* ln [$Na^+$] to obtain the slope *SK* value, which is essential for polyelectrolyte analysis. The enthalpy and entropy changes of the DNA binding were obtained from the van't Hoff plot of ln $K_b$ *versus* 1/*T* (*T* in Kelvin). Each measured point was the average value of at least three separate measurements with a relative standard deviation (RSD) normally less than 15 %.

## Result and Discussion

*Absorption spectral features*

The absorption spectra are the most common means to observe the interaction between metal complexes and DNA. Upon the DNA interaction, the absorption spectra of metal complexes generally exhibit significant hypochromism and red shift due to the $\pi$–$\pi$ stacking interaction between the aromatic chromophore ligand of the metal complexes and the base pairs of DNA. The absorption spectral features of racemic $[Fe(DMP)_3]^{2+}$ and $[Fe(TMP)_3]^{2+}$ in the presence of increasing amounts of ct-DNA in 5 mM Tris-HCl buffer containing 50 mM NaCl are presented in Fig. 2. The spectra were characterized by the intense $d \rightarrow \pi^*$ MLCT band of iron(II) complexes observed in the visible region of 400 – 650 nm. The addition of increasing amounts of ct-DNA to the racemic solution of the iron(II) complexes resulted in a hypochromicity (H: *ca.* 25 – 40 %) and a small red shift ($\Delta\lambda_{max}$ = 1 – 3 nm) of the absorption maxima centered at 512 and 503 nm for $[Fe(DMP)_3]^{2+}$ and $[Fe(TMP)_3]^{2+}$, re-

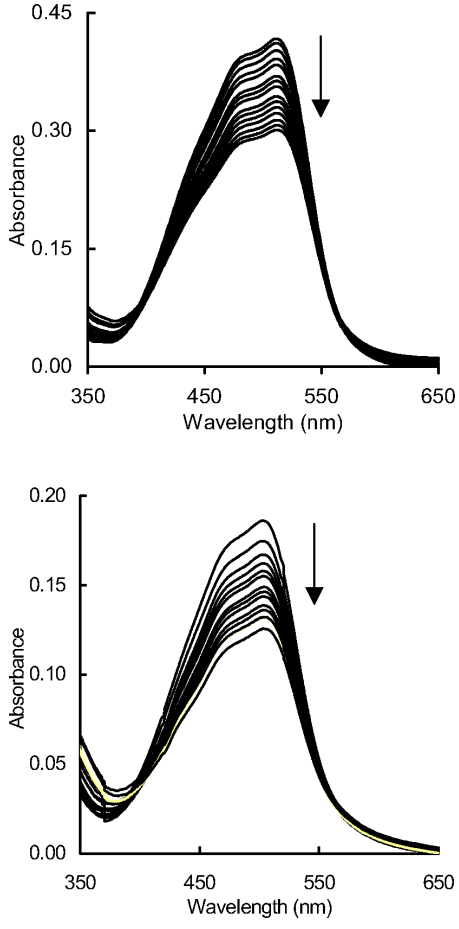


Fig. 2. Absorption spectral changes of 26.5 μM $[Fe(DMP)_3]^{2+}$ (upper) and 10.5 μM $[Fe(TMP)_3]^{2+}$ (lower) in 5 mM Tris-HCl buffer at pH 7.2 and 50 mM NaCl at 25 °C upon increasing addition of ct-DNA ($R = \infty - 0.05$). The arrows show hypochromism in metal-to-ligand charge transfer (MLCT) bands.

Table 1. Hypochromicity (H), red shift and equilibrium binding constant ($K_b$) for $[Fe(DMP)_3]^{2+}$ binding to calf thymus DNA in 5 mM Tris-HCl buffer (pH = 7.2) at 25 °C and various concentrations of NaCl[a].

| [NaCl](M $L^{-1}$) | H ( %), 511 nm | $\Delta\lambda_{max}$ (nm) | $K_b$ ($M^{-1}$ $bp^{-1}$) | $K_t^0$ ($M^{-1}$ $bp^{-1}$) | $K_t^0$ / $K_b$ / % |
|---|---|---|---|---|---|
| 0.005 | 30.1 | +2.0 | $5.65 \times 10^4$ | $1.28 \times 10^4$ | 22.7 |
| 0.025 | 29.4 | +2.0 | $3.63 \times 10^4$ | $1.26 \times 10^4$ | 34.7 |
| 0.050 | 27.9 | +1.5 | $2.99 \times 10^4$ | $1.25 \times 10^4$ | 41.8 |
| 0.075 | 24.2 | +1.0 | $2.73 \times 10^4$ | $1.26 \times 10^4$ | 46.2 |
| | | | | Average of $K_t^0 = 1.263 \pm 0.013$ ($\times 10^4$) | |

[a] DMP = 4,7-dimethyl-1,10-phenanthroline; hypochromicity was determined at $R$ ([complex]/[ct-DNA]) = 0.1; $K_b$ was calculated according to Eq. 1; $K_t^0$ is the non-electrostatic contribution to the overall equilibrium binding constant ($K_b$) and evaluated by Eq. 2 using a $Z$ value of 0.3083 (Fig. 4).

Table 2. Hypochromicity (H), red shift and equilibrium binding constant ($K_b$) for $[Fe(TMP)_3]^{2+}$ binding to calf thymus DNA in 5 mM Tris-HCl buffer (pH = 7.2) at 25 °C and various concentrations of NaCl[a].

| [NaCl] (M $L^{-1}$) | H ( %), 511 nm | $\Delta\lambda_{max}$ (nm) | $K_b$ ($M^{-1}$ $bp^{-1}$) | $K_t^0$ ($M^{-1}$ $bp^{-1}$) | $K_t^0$ / $K_b$ / % |
|---|---|---|---|---|---|
| 0.005 | 38.8 | +3.0 | $19.2 \times 10^4$ | $3.34 \times 10^4$ | 17.4 |
| 0.025 | 35.3 | +3.0 | $11.9 \times 10^4$ | $3.39 \times 10^4$ | 28.5 |
| 0.050 | 32.5 | +2.0 | $8.73 \times 10^4$ | $3.10 \times 10^4$ | 35.5 |
| 0.100 | 30.9 | +1.0 | $7.50 \times 10^4$ | $3.29 \times 10^4$ | 43.9 |
| | | | | Average of $K_t^0 = 3.280 \pm 0.128$ ($\times 10^4$) | |

[a] TMP = 3,4,7,8-tetramethyl-1,10-phenanthroline; hypochromicity was determined at $R$ ([complex]/[ct-DNA]) = 0.1; $K_b$ was calculated according to Eq. 1; $K_t^0$ is the non-electrostatic contribution to the overall equilibrium binding constant ($K_b$) and evaluated by Eq. 2 using a $Z$ value of 0.3656 (Fig. 4).

spectively, suggesting that both iron(II) complexes interact strongly with double helical ct-DNA. Isobestic points near 400 and 550 nm are also observed for the absorption spectra of the two iron(II) complexes, indicating that there is an equilibrium state between iron(II) complexes and ct-DNA. As predicted, the extent of hypochromicity and red shift upon binding of the two iron(II) complexes to ct-DNA decreases with the salt concentration in the solution (Tables 1 and 2), suggesting that electrostatic interaction is involved in the binding event. As compared to their parent complex, $[Fe(phen)_3]^{2+}$, which is known to interact electrostatically with DNA, substitution at the 4 and 7 positions of the phen ligand by two methyl groups as in $[Fe(DMP)_3]^{2+}$ significantly increases the percentage of hypochromicity from 15 % [29] to 28 % at $R$([complex]/[DNA]) = 0.1 upon binding to ct-DNA. Further substitution by two more methyl groups at the 3 and 8 position as in $[Fe(TMP)_3]^{2+}$ consistently increases the hypochromicity by 32.5 %, suggesting that there exists stronger interaction with ct-DNA. However, this large hypochromicity is not accompanied by any red shift, *i. e.* by any significant increase in wavelength of the absorption maxima upon binding of the two iron(II) complexes to ct-DNA. This phenomenon may be interpreted as follows. Unlike their parent complex, the two iron(II) complexes are not fully involved in the electrostatic interaction, because the addition of methyl subsituents to the phen ligand allows the ligand to more deeply penetrate between the DNA base pairs, resulting in partial intercalation of one ligand into the base pairs of ct-DNA. This interpretation is also supported by the pronounced hypochromicity in the MLCT bands. The absence of a significant red shift may be attributed to the fact that unlike planar aromatic substituents, methyl groups can not be involved in $\pi$–$\pi$ stacking interactions and thus do not affect the $\pi$–$\pi^*$ transition energy. This consequence is further supported by the data obtained from salt-dependence studies of the binding constants.

### *Salt-concentration dependence of equilibrium binding constants*

Fig. 3 shows an example of typical plots of [ct-DNA]/($\varepsilon_A - \varepsilon_F$) *versus* [ct-DNA] for the determination of the binding constant ($K_b$) of $[Fe(DMP)_3]^{2+}$ to ct-DNA at 25 and 50 mM NaCl based on Eq. 1. Detailed results of the determination of $K_b$ in the salt-concentration range of 0.005 – 0.100 M for $[Fe(DMP)_3]^{2+}$ and $[Fe(TMP)_3]^{2+}$ are collected in Tables 1 and 2, respectively. The salt concentrations of 0.005 – 0.100 M were selected in this study because the polyelectrolyte theory which will be used for subsequent analysis to calculate the non-electrostatic binding constant and separate the binding free energy

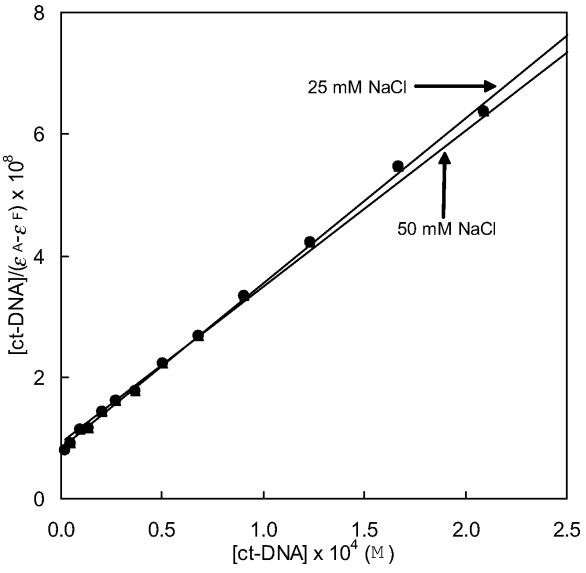


Fig. 3. Typical plots of [ct-DNA]/($|\varepsilon_A - \varepsilon_F|$) *vs.* [ct-DNA] for the determination of the equilibrium binding constant ($K_b$) based on Eq. 1 of the ct-DNA binding of $[Fe(DMP)_3]^{2+}$ at various salt concentrations.

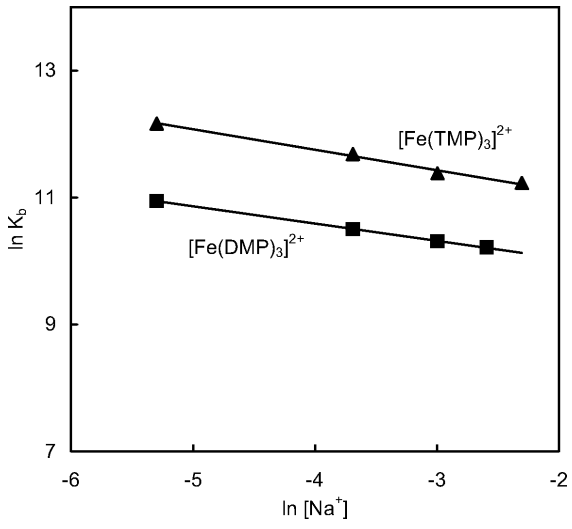


Fig. 4. Salt-concentration dependence of the equilibrium binding constant ($K_b$) for the binding of $[Fe(DMP)_3]^{2+}$ and $[Fe(TMP)_3]^{2+}$ to ct-DNA. The slope of these linear regressions corresponds to *SK* values presented in Table 3.

change into its electrostatic and non-electrostatic portion is based on limiting laws that are strictly applicable to salt concentrations of lower than 0.100 M [30]. The plot of the salt-dependence of the binding constant, ln $K_b$ *versus* ln $[Na^+]$ for the ct-DNA binding of $[Fe(DMP)_3]^{2+}$ and $[Fe(TMP)_3]^{2+}$, is given in Fig. 4.

It is obvious from Fig. 4 that the equilibrium binding constant decreases as the salt concentration in the solution increases. This can be attributed to the stoichiometric counter ion release following the binding of positively charged complexes, *i. e.* iron(II) complexes in this case [31], suggesting that in addition to partial intercalation an electrostatic interaction is involved in the DNA binding event. By using the slope value of the linear fitting obtained from Fig. 4, the non-electrostatic binding constant ($K^0{}_t$) at various concentrations of NaCl can be calculated on the basis of the following Eq. 2 for polyelectrolytes [31]:

$$\ln K_b = \ln K^0{}_t + Z\xi^{-1}\{\ln(\gamma_\pm \delta)\} + Z\psi(\ln[M^+]) \quad (2)$$

where $Z\psi$ is obtained from the slope of the straight line in Fig. 4, $Z$ is the partial charge on the binding ligand (iron(II) complexes) involved in the DNA interaction as predicted by polyelectrolyte theory, $\psi$ the fraction of counterions associated with each DNA phosphate ($\psi$ = 0.88 for double-stranded B-form DNA), $\gamma_\pm$ the mean activity coefficient at a salt concentration $[M^+]$, and the remaining terms are constants for double-stranded DNA in B-form, *i. e.* $\xi$ = 4.2 and $\delta$ = 0.56. Results of the calculations are summarized in Tables 1 and 2 for $[Fe(DMP)_3]^{2+}$ and $[Fe(TMP)_3]^{2+}$ along with the percentage ratio of $K^0{}_t$ to the total binding constant ($K_b$) at various concentrations of $M^+$ ($Na^+$). These $K^0{}_t$ parameters can be taken as a measure for how much the non-electrostatic forces stabilize the DNA binding of iron(II) complexes. As can be seen from Tables 1 and 2, the values of $K^0{}_t$ are constant throughout the employed concentration of NaCl with average values of $1.263(\pm 0.013) \times 10^4$ and $3.280(\pm 0.128)$ $M^{-1}$ bp for $[Fe(DMP)_3]^{2+}$ and $[Fe(TMP)_3]^{2+}$, respectively, because $K^0{}_t$ is the non-electrostatic binding constant which is independent of salt concentration. Although the values of $K^0{}_t$ are constant throughout the concentration of salt, the $K_b$ values decrease with the concentration of salt. Therefore, the percentage of $K^0{}_t$ in $K_b$ increases significantly and reaches maximum values of 46.4 % for $[Fe(DMP)_3]^{2+}$ at $[Na^+]$ = 0.075 M and 43.9 % for $[Fe(TMP)_3]^{2+}$ at $[Na^+]$ = 0.100 M. In other words, more than 40 % of the DNA interaction is due to non-electrostatic processes, *e. g.* intercalation. It can be predicted that at higher concentrations of salt, *e. g.* under physiological conditions ($Na^+ \approx 0.200$ M), the non-electrostatic forces would be the major driving forces in the DNA binding of the iron(II) complexes.

Table 3. Thermodynamic parameters for the binding of iron(II) complexes of 1,10-phenanthroline (phen) and its derivatives to calf thymus DNA[a].

| Iron(II) complexes | $K_b$ $10^3$ M$^{-1}$ bp$^{-1}$ | $K_t^0$ $10^3$ M$^{-1}$ bp$^{-1}$ | $SK$ kJ mol$^{-1}$ | $\Delta H^0$ kJ mol$^{-1}$ | $T\Delta S^0$ kJ mol$^{-1}$ | $\Delta G^0$ kJ mol$^{-1}$ | $\Delta G_{pe}^0$ kJ mol$^{-1}$ | $\Delta G_t^0$ kJ mol$^{-1}$ |
|---|---|---|---|---|---|---|---|---|
| $[Fe(phen)_3]^{2+}$ [b] | 4.68 | 0.205 (4.4) | 0.97 | +30.1 | +51.0 | −20.9 | −7.2 | −13.7 (65.6) |
| $[Fe(DMP)_3]^{2+}$ | 29.9 | 12.5 (41.7) | 0.27 | +38.3 | +63.8 | −25.6 | −2.0 | −23.6 (92.1) |
| $[Fe(TMP)_3]^{2+}$ | 87.3 | 31.0 (35.4) | 0.32 | –[d] | –[d] | −28.2 | −2.4 | −25.8 (91.5) |
| $[Fe(phen)_2(dppz)]^{2+}$ [c] | 157 | 32.4 (20.6) | 0.49 | +28.1 | +57.7 | −29.7 | −3.6 | −26.1 (87.9) |

[a] In 50 mM NaCl and 5 mM Tris-HCl buffer (pH = 7.2) at 25 °C. $\Delta G^0$ is the binding free energy change calculated by using Eq. 3. $SK$ ($Z\psi$) is the absolute value of the slope obtained from the plots of Fig. 4. $\Delta G_{pe}^0$ and $\Delta G_t^0$ are the electrostatic and thermodynamic binding free energy contributions to overall $\Delta G^0$, respectively; [b] $\Delta G_{pe}^0$ values were calculated using Eq. 5 at $[Na^+]$ = 50 mM, and $\Delta G_t^0$ was calculated by the difference [29]; [c] taken from our previous study [22, 33]. The figures in parentheses for $K_t^0$ and $\Delta G_t^0$ correspond to the percentage of $K_t^0$ in $K_b$ and of $\Delta G_t^0$ to $\Delta G^0$, respectively. dppz = dipyrido[3,2-*a*:2′,3′-*c*]phenazine; [d] could not be determined due to low solubility.

A comparison of $K^0{}_t$ reveals that the contribution of $K^0{}_t$ to $K_b$ increases from 5.44 % at $[Na^+]$= 50 mM in $[Fe(phen)_3]^{2+}$ [22] to 41.8 % and 35.5 % in $[Fe(DMP)_3]^{2+}$ and $[Fe(TMP)_3]^{2+}$, respectively, at the same ionic strength, and thus the substitution by methyl groups in the ligand improves significantly the non-electrostatic forces of DNA binding. The slightly lower ratio of $K^0{}_t$ to $K_b$ for $[Fe(TMP)_3]^{2+}$ as compared to that for $[Fe(DMP)_3]^{2+}$ may be attributed to the steric effect resulting from further substitution at the 3 and 8 position of the phen ligand by two more methyl groups. The substitution at these positions causes the ancillary/non-intercalative ligands to become too bulky and to clash with the phosphate backbones, so that the penetration of the intercalative ligand in $[Fe(TMP)_3]^{2+}$ is hindered. However, it should be noted that the actual value of $K^0{}_t$ for $[Fe(TMP)_3]^{2+}$ is still 2.6 times higher than that for $[Fe(DMP)_3]^{2+}$, although its ratio of $K^0{}_t$ to $K_b$ is slightly lower than that of $[Fe(DMP)_3]^{2+}$.

The effect of methyl substitution on the DNA binding can be further evaluated by separating the binding free energy change ($\Delta G^0$) for the interaction of iron(II) complexes with ct-DNA into their electrostatic ($\Delta G_{pe}^0$) and non-electrostatic ($\Delta G_t^0$) contributions at a given concentration of salt [32]. In Table 3 are collected the thermodynamic parameters for the binding of $[Fe(DMP)_3]^{2+}$ and $[Fe(DMP)_3]^{2+}$ to ct-DNA in 0.050 M NaCl along with those of other iron(II) complexes for the purpose of comparison. The overall binding free energy changes listed in Table 3 were calculated from the standard Gibbs relation (Eq. 3):

$$\Delta G^0 = -RT \ln K_b \tag{3}$$

where R is the gas constant and $T$ the temperature in Kelvin. The salt-concentration dependence of the binding constant (Fig. 4) gives the slope, $SK$ (Eq. 4):

$$SK = \delta \log K_b / \delta \log[Na^+] = -Z\psi \tag{4}$$

The $SK$ value can then be used to calculate the polyelectrolyte/electrostatic contribution of the free energy change ($\Delta G_{pe}^0$) to the overall free energy change ($\Delta G^0$) at a given NaCl concentration by Eq. 5 [32, 33]:

$$\Delta G_{pe}^0 = (SK)RT \ln[Na^+] \tag{5}$$

The difference between the Gibbs free energy change ($\Delta G^0$) and $\Delta G_{pe}^0$ defines the non-electrostatic free energy change ($\Delta G_t^0$, Eq. 6):

$$\Delta G_t^0 = \Delta G^0 - \Delta G_{pe}^0 \tag{6}$$

The quantity $\Delta G_t^0$ corresponds to the portion of the binding free energy change, which is independent of salt concentrations and contains a minimal contribution from polyelectrolyte effects such as coupled ion release. Although the iron(II) complexes used in this study formally have a +2 charge, the partial charges involved in the interaction are not always the same due to the difference in structure and complexity of the iron(II) complexes. Therefore, before the effect of methyl substitution on the thermodynamic parameters of the DNA binding of iron(II) complexes is compared, it is necessary to first calculate $K^0{}_t$ and dissect the values of $\Delta G^0$ into $\Delta G_{pe}^0$ and $\Delta G_t^0$ and then to use the value of $\Delta G_t^0$ for evaluating the effect of substitution on the total binding free energy change. This approach is justified because the variation in the quantity of $\Delta G_t^0$ of the DNA binding of iron(II) complexes would be largely due to the variation in the substitution which has been made in the iron(II) complexes.

*Temperature dependence of equilibrium binding constants*

The determination of equilibrium binding constants at various temperatures allows us to evaluate enthalpy ($\Delta H^0$) and entropy ($\Delta S^0$) changes with respect to the DNA binding of iron(II) complexes by using the van't Hoff plot of ln $K_b$ *versus* $1/T$ with the assumption that $\Delta H^0$ and $\Delta S^0$ are independent of temperature within the limited temperature range. Such studies have been exceptionally rare although there have been many reports about the interaction of metal complexes with DNA [30]. In fact, the thermodynamic parameters such as free energy, enthalpy and entropy changes of DNA-metal complex interactions are indispensable for a detailed understanding of the driving forces responsible for the interaction between metal complexes and DNA. Therefore, we have determined the binding constant ($K_b$) for the ct-DNA binding of $[Fe(DMP)_3]^{2+}$ at various temperatures, *i. e.* 11, 18, 25 and 32 °C by spectrophotometric titration and analyzed the $K_b$ by using Eq. 1. Due to the low solubility of $[Fe(TMP)_3]^{2+}$, similar experiments to those for $[Fe(DMP)_3]^{2+}$ could not be performed at various temperatures. In Fig. 5, the typical van't Hoff plot of ln $K_b$ *versus* $1/T$ is shown for the DNA binding of $[Fe(DMP)_3]^{2+}$ in the corresponding temperature range. The enthalpy change ($\Delta H^0$) of the DNA binding reaction is immediately obtained from the slope of the plot which is equal to $-\Delta H^0/R$ where R = gas constant, while the entropy of the reaction may also be obtained from the Y-intercept which is equal to $\Delta S^0/R$. The results of the calculations are summarized in Table 3 together with those of other iron(II) complexes for the purpose of comparison.

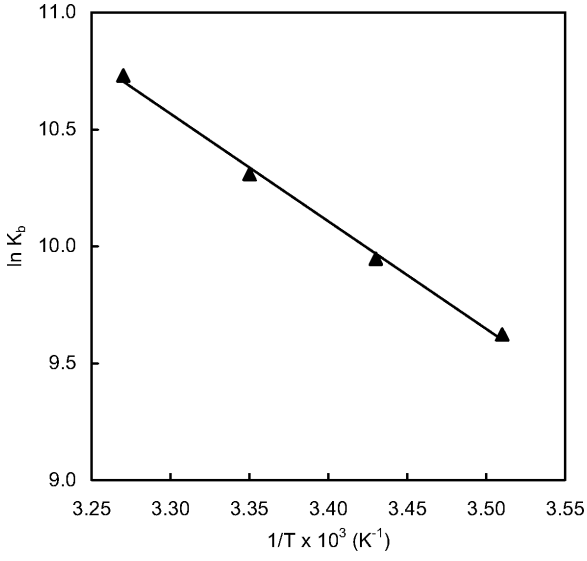


Fig. 5. The van't Hoff plot of ln $K_b$ *vs.* $1/T$ for the binding of $[Fe(DMP)_3]^{2+}$ to ct-DNA. The slope and Y-intercept of the plot are equal to $-\Delta H^0/R$ and $\Delta S^0/R$, respectively.

The striking observation of Fig. 5 and Table 3 is that the DNA binding reaction of $[Fe(DMP)_3]^{2+}$ is endothermic because the enthalpy change is positive, and from the Gibbs relation: $\Delta G^0 = \Delta H^0 - T\Delta S^0$, we immediately recognize that the DNA binding reaction of $[Fe(DMP)_3]^{2+}$ is entropically driven. This finding is quite common for the DNA binding of metal complexes of phen and its derivatives [30, 32, 33] (see also Table 3). The possible reason for the entropically driven DNA binding of these types of metal complexes has been thoroughly discussed from the viewpoint of molecular interactions [30, 33]. In general, the counterion release, change in hydration and hydrophobic interaction, which are caused by the transfer of binding ligands from the aqueous solvent to the interior of the DNA helix, are the most relevant processes that yield the entropically driven DNA binding.

It is also clearly seen from Table 3 that the free energy change ($\Delta G^0$) in the DNA binding of $[Fe(DMP)_3]^{2+}$ is more negative by 4.7 kJ mol$^{-1}$ as compared to that of its parent complex, $[Fe(phen)_3]^{2+}$, which is known to be more electrostatically bound to DNA. This additional force is undoubtedly due to the substitution of hydrogen atoms at the 4 and 7 positions of the phen by methyl groups. Further substitution by methyl groups at the 3 and 8 positions as in $[Fe(TMP)_3]^{2+}$ consistently brings about additional binding free energy of 2.6 kJ mol$^{-1}$ to afford more favorable DNA binding. However, these values are still less favorable for the DNA binding, *i. e.* by 4.1 kJ mol$^{-1}$ and 1.5 kJ mol$^{-1}$, than that of $[Fe(phen)_2(dppz)]^{2+}$, which is confirmed to interact with DNA in an intercalation mode *via* the dppz ligand [33]. The moderate values of $\Delta G^0$ for the DNA binding of $[Fe(DMP)_3]^{2+}$ and $[Fe(TMP)_3]^{2+}$ are between those of the electrostatic DNA binder $[Fe(phen)_3]^{2+}$ and the DNA intercalator $[Fe(phen)_2(dppz)]^{2+}$, indicating that the two iron(II) complexes interact with ct-DNA through electrostatic binding and partial intercalation as suggested previously.

*Influence of methyl substitution on the electrostatic stabilization of DNA binding*

Eq. 4 defines that the slope of the linear regression line in Fig. 4, *SK*, is equal to $-Z\psi$ where $Z$ corresponds to the partial charge of the interacting ligand involved in the DNA binding. Since the iron(II) complexes used in this study have the formal charge of $+2$, the value of *SK* for the two complexes theoretically would be $2 \times 0.88 = 1.76$ ($Z = SK/\psi$, or $SK = Z \times \psi$, where $\psi = 0.88$ for B-form DNA). However, an examination of Table 3 for the *SK* values reveals that the experimental *SK* values (0.27 – 0.97) for all iron(II) complexes listed are much lower as compared to the theoretical one (1.76). Although the lower *SK* value is quite common for octahedral metal complexes of phen and its derivatives [22, 34, 35], no plausible explanation has been suggested so far with respect to the structure of the metal complexes and/or the location of positive charge on the binding ligand. In our previous report [22], we have suggested that the lower *SK* for the DNA binding of octahedral metal complexes may be induced by the fact that the positive charge of the complexes is situated at the central metal ion which is distant from the binding site due to the bulkiness of the ligand attached to it. As a result, the positive charge of the metal complexes is not fully involved in the DNA binding. Moreover, as the central metal ion is coordinated by three ligands at different directions, the positive charge of the central metal ion would also be diminished or transferred to three different directions. Indeed, only one ligand coordinated to the central metal ion is directly involved in close contact with the DNA base pairs due to the steric hindrance. Therefore, it is quite easy to understand that the charge or *SK* value of octahedral metal complexes with bulkier ligands is normally much lower than the expected value.

The influence of methyl substitution on the electrostatic stabilization for the DNA binding of iron(II) complexes can be evaluated by comparing the value of $\Delta G^0_{pe}$, or more precisely $\Delta\Delta G^0_{pe}$ (Table 4), *i. e.* the difference between the electrostatic portion of the binding free energy change for the DNA binding of iron(II) complexes studied and that for their parent complex $[Fe(phen)_3]^{2+}$. It can be seen from Tables 3 and 4 that the electrostatic contribution to the stabilization of the DNA binding of iron(II) complexes with the formal charge of $+2$ varies considerably as indicated by their $\Delta G^0_{pe}$ values, *e. g.* from $-2.0$ kJ mol$^{-1}$ to $-7.2$ kJ mol$^{-1}$ in 50 mM NaCl depending on the attached ligands. Nevertheless, the $\Delta\Delta G^0_{pe}$ for all substituted complexes has a positive sign relative to that of the parent complex $[Fe(phen)_3]^{2+}$, indicating that methyl substitution of the phen ligand leads electrostatically to a less favorable state for DNA binding. As already explained before, the substitution makes the ligand bulkier to yield steric effects, and the positive charge at the central metal ion becomes more distant from the binding site. Moreover, a close examination of Table 4 reveals that the substitution gives rise to the loss of binding free energy of 4.0 – 5.0 kJ mol$^{-1}$ making it electrostatically less favorable for DNA binding. However, we will see later that the loss of electrostatic binding free energy due to this substitution is compensated by the large quantity of non-electrostatic binding free energy change so that the net DNA-binding free energy change for all substituted iron(II) complexes is much more favorable for the DNA binding as compared to that for the parent complex $[Fe(phen)_3]^{2+}$. This tendency is consistent with our previous conclusion that the binding mode of iron(II) complexes to DNA is shifted from an electrostatic interaction as in $[Fe(phen)_3]^{2+}$to a mixture of electrostatic interaction and partial intercalation in substituted iron(II) complexes.

Table 4. Energetics cost of group substitution in iron(II) complexes of 1,10-phenanthroline and its derivatives[a].

| Complex | Substitution | $\Delta\Delta G^0_{pe}$ (kJ mol$^{-1}$) | $\Delta\Delta G^0_t$ (kJ mol$^{-1}$) |
|---|---|---|---|
| $[Fe(phen)_3]^{2+}$ | reference compound | 0.00 | 0.00 |
| $[Fe(DMP)_3]^{2+}$ | two H by two methyl | +5.20 | −9.90 |
| $[Fe(TMP)_3]^{2+}$ | four H by four methyl | +4.80 | −12.1 |
| $[Fe(phen)_2(dppz)]^{2+}$ | one phen by one dppz | +4.30 | −12.4 |

[a] The quantity of $\Delta\Delta G^0_{pe}$ and $\Delta\Delta G^0_t$ refer respectively to the difference in electrostatic and non-electrostatic binding free energy changes relative to those of the reference compound $[Fe(phen)_3]^{2+}$. The negative sign of $\Delta\Delta G^0$ indicates more favorable DNA binding, while the positive one corresponds to less favorable DNA binding.

*Effect of methyl substitution on the non-electrostatic stabilization of DNA binding*

The difference between the non-electrostatic portion of the DNA binding free energy change for the iron(II) complexes and that for the parent iron(II) complex $[Fe(phen)_3]^{2+}$ ($\Delta\Delta G^0_t$) can be used to estimate the binding free energy contribution of specific substituents of the ligand to the stabilization of DNA binding. The calculated $\Delta\Delta G^0_t$ values for $[Fe(DMP)_3]^{2+}$

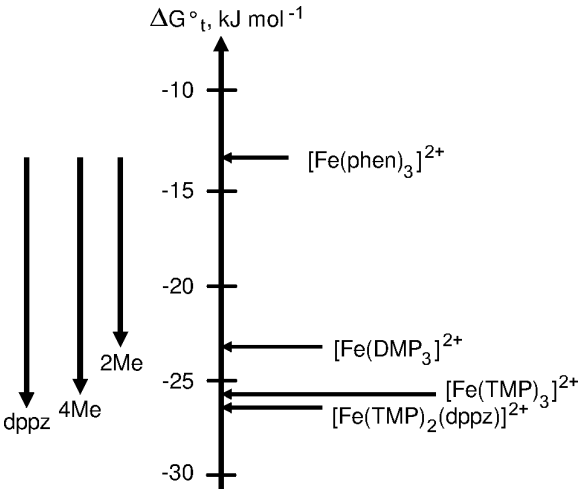


Fig. 6. Free energy diagram showing the effect of methyl substitution on the non-electrostatic portion of the binding free energy change. The magnitude of $\Delta G^0_t$ (kJ mol$^{-1}$) is shown on the axis. The arrows on the left side indicate the magnitude of the non-electrostatic free energy difference ($\Delta\Delta G^0_t$) resulting from methyl substitution.

and $[Fe(TMP)_3]^{2+}$ are collected along with those of other iron(II) complexes in Table 4 and shown graphically in Fig. 6. By assuming that the binding free energy contributions resulting from conformational changes and from the loss of rotational freedom due to DNA binding are the same for all the complexes studied, it can be justified that the $\Delta\Delta G^0_t$ value of a particular complex reflects primarily the differences in its molecular interactions within the binding site, which are brought about by the variation of substituents on the ligand. In contrast to $\Delta\Delta G^0_{pe}$, a negative sign of $\Delta\Delta G^0_t$ is observed in all cases in Table 4, suggesting that each methyl substitution on the phen ligand gives rise to more favorable DNA binding with respect to $[Fe(phen)_3]^{2+}$. In other words, although methyl substitution of the phen ligand slightly destabilizes the DNA binding from the viewpoint of electrostatic force, it has been proved here that the substitution strongly stabilizes the DNA binding of the iron(II) complexes in terms of non-electrostatic processes.

The most interesting outcome of this study is to have evaluated quantitatively the contribution of some substituents such as methyl groups and phenazine moieties to the binding free energy. Although the role of planar heterocyclic moieties in enhancing the DNA binding affinity has been well discussed [1, 2], any quantitative evaluation of the energy contribution of non-planar moieties like methyl substituents has been hardly attempted. The detailed examination of $\Delta\Delta G^0_t$ in Table 4 has revealed that the energetic stabilization of 9.90 kJ mol$^{-1}$ for the DNA binding is induced by the substitution of two hydrogen atoms at the 4 and 7 positions in the phen by two methyl groups. This value represents more than 47 % of the total binding free energy change of $[Fe(phen)_3]^{2+}$ to DNA and corresponds to about a 60-fold increase in the non-electrostatic binding constant ($K^0{}_t$). Such a large stabilization in DNA binding suggests that the binding mode of iron(II) complexes to DNA is also shifted from a mainly electrostatic interaction for $[Fe(phen)_3]^{2+}$to mixtures between electrostatic interaction and partial intercalation for $[Fe(DMP)_3]^{2+}$as previously concluded. Further substitution of two hydrogen atoms at the 3 and 8 position of the phen by two methyl groups like in $[Fe(TMP)_3]^{2+}$ consistently affords 12.1 kJ mol$^{-1}$ of more favorable free energy change for DNA binding, which is slightly lower than that obtained for $[Fe(phen)_2(dppz)]^{2+}$. This quantity is 3.2 kJ mol$^{-1}$ larger than that of $[Fe(DMP)_3]^{2+}$ and corresponds to more than 57 % of the total binding free energy change obtained from its parent complex (Fig. 6). The reason for the non-linear increase in non-electrostatic binding free energy for the second substitution has been discussed previously and attributed mainly to the steric effect brought about by the bulkiness of the ligand. In conclusion, we have demonstrated here that although methyl substitution destabilizes slightly the DNA binding of the iron(II) complexes from the viewpoint of electrostatic forces, the substitution contributes substantially to the total binding free energy change and thus significantly stabilizes the binding of the iron(II)-phenanthroline complexes to DNA through non-electrostatic forces.

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