DNA-Binding Properties of Iron(II) Mixed-Ligand Complexes Containing 1,10-Phenanthroline and Dipyrido[3,2-*a*:2',3'-*c*]phenazine

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An iron(II) mixed-ligand complex with 1,10-phenanthroline (phen) and dipyrido[3,2-*a*:2',3'-*c*]phenazine (dppz), $[Fe(phen)_2(dppz)]^{2+}$, has been synthesized. The DNA-binding properties of the mixed-ligand complex have been studied in terms of equilibrium binding constant, thermodynamic parameter, thermal denaturation as well as Pfeiffer effect upon binding to DNA. The spectrophotometric titration of $[Fe(phen)_2(dppz)]^{2+}$ with calf thymus DNA (ct-DNA) has shown that the iron(II) mixed-ligand complex binds effectively to ct-DNA in an intercalation mode as indicated by remarkable hypochromicity (*ca.* 36%) and moderate bathochromic shift (8 nm) of the absorption spectra. This intercalative mode is supported by a significant increase ($\Delta T_m = 21$ °C) in the melting temperature (T_m) of ct-DNA at R([complex]/[ct-DNA]) = 1.5. The binding of $[Fe(phen)_2(dppz)]^{2+}$ to ct-DNA is entropically driven as characterized by a positive enthalpy change and a large negative $T\Delta S$ term. An intense CD signal in the UV and visible region develops upon addition of ct-DNA to the racemate solution of $[Fe(phen)_2(dppz)]^{2+}$. This has revealed that a shift in diastereomeric inversion equilibrium takes place in the solution to yield an excess of one enantiomer of the DNA-iron(II) complex (Pfeiffer effect). The striking resemblance of the CD spectral profiles to those of the corresponding Δ-enantiomer indicates that Δ-$[Fe(phen)_2(dppz)]^{2+}$ is preferentially bound to ct-DNA.

Key words: Iron(II), Mixed Ligand Complex, Phenanthroline, Dppz, DNA Binding

Introduction

In the past decade there has been considerable interest in interactions of cationic inert complexes of transition metal ions with nucleic acids [1 – 5]. A variety of cationic metal complexes have been utilized to develop novel probes of DNA [6 – 8], new therapeutic agents which can recognize and cleave DNA [9 – 15], and DNA-mediators of electron transfer reactions [4, 16 – 17]. Recently, there has been more interest in DNA probing based on the development of a DNA chip used for genetic diagnosis. In design and synthesis of new metallointercalators for their many potential DNA-related applications, it is important to understand the factors that govern the DNA binding of the intercalators [1, 3]. Among the metal complexes studied, the complexes with planar aromatic ligands such as 1,10-phenanthroline (phen) and dipyrido[3,2-*a*:3',2'-*c*]phenazine (dppz) that bind to DNA have attracted intense scrutiny because of their environmentally sensitive photophysics. Although lacking luminescence in aqueous solution, they give rise to intense luminescence when intercalated into the DNA base pairs, termed as molecular "light switch" for DNA [8, 18 – 19]. This luminescence characteristic accompanied by high binding affinity for DNA permits the application of the complexes as a non-radioactive probe of double-stranded DNA at an analytical level. Furthermore, these complexes also have many convenient features to be used for DNA-binding studies, including the easy coordination of the ligands to the metal ions in a controlled manner, strong electronic absorption in the visible region due to metal-to-ligand charge transfer (MLCT) or $\pi - \pi^*$ transitions of the ligands, and strong luminescence. These features provide a convenient handle for monitoring the process and mecha-

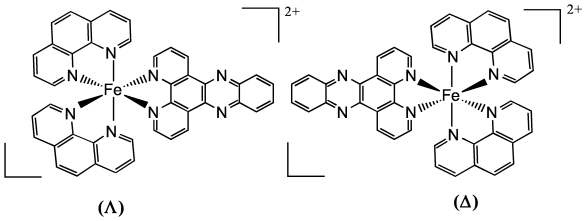

Fig. 1. Chemical structure of iron(II) mixed-ligand complex containing phen and dppz ligands.

nism of the DNA binding event by spectroscopic methods [20].

Unlike the metal complexes of phen or bpy (bpy=2,2'-bipyridine), the mixed-ligand metal complexes of phen and dppz have been unambiguously shown to intercalate into the base pairs of double helical DNA [8, 18 – 23]. The mixed-ligand ruthenium(II) complex $[Ru(phen)_2(dppz)]^{2+}$ has so far been most extensively used for the DNA-binding studies and reported to be an avid binder of DNA [18 – 23]. However, DNA-binding studies of mixed-ligand metal complexes with the central metal ions other than ruthenium(II) have attracted much less attention except for recent reports on rhodium [7], osmium(II) [8], cobalt(II) and nickel(II) [10] and rhenium(I) [11] complexes. Clearly, further studies of the mixed-ligand complexes of central metal ions other than the above mentioned metal ions are needed to investigate the influence of the changes in geometry, charge, spin state, redox potential *etc.* on the DNA-binding process and mechanism of the mixed-ligand complexes. These studies also serve as complementary ones to those of mixed-ligand complexes containing ruthenium(II) and other metal ions mentioned above. In the present study, the DNA-binding properties of bis(1,10-phenanthroline)(dipyrido[3,2-*a*:3',2'-*c*]phenazine)iron(II), $[Fe(phen)_2(dppz)]^{2+}$ (Fig. 1) have been fully characterized by means of equilibrium binding constant (K_b), melting temperature (T_m) of double helical DNA upon binding to the metal complex, thermodynamic parameter of the DNA binding, and enantioselectivity of the DNA binding of the iron(II) mixed-ligand complex.

Most studies of the DNA binding of metal complexes have been concerned with establishing their binding mode and with the possible structure of DNA complexes. Apart from the determination of K_b, the thermodynamics of the DNA binding has been hardly studied in detail. One of the purposes of the present study is to determine a thermodynamic profile, *i.e.* free energy change (ΔG), enthalpy change (ΔH) and entropy change (ΔS) of the DNA binding of the iron(II) mixed-ligand complex using the van't Hoff plot. These thermodynamic data allowed us to evaluate the enthalpic and entropic contributions to ΔG° of the DNA binding of metal complexes [24 – 28]. Moreover, a difference in biological activities between the enantiomers of optically active metal complexes has been noted in many examples such as toxicity and drug efficiency [29, 30]. In order to better understand such effects at the level of molecular interactions, we have also examined enantiomeric effects on the DNA binding of $[Fe(phen)_2(dppz)]^{2+}$ by CD spectropolarimetry. This kind of examination will give an answer to a question which enantiomer, *i.e.* Δ or Λ, is well-fitted to the structure of the right-handed double helical calf-thymus DNA (ct-DNA). The binding mode of $[Fe(phen)_2(dppz)]^{2+}$ to ct-DNA has been deduced from the UV-visible spectral profile of $[Fe(phen)_2(dppz)]^{2+}$ in the absence and presence of ct-DNA and from the data of thermal denaturation experiments of ct-DNA in the absence and presence of $[Fe(phen)_2(dppz)]^{2+}$. The binding of an intercalative drug to DNA has been classically characterized by hypochromism and red shift of UV-visible spectra of metal complexes as well as by the increase in melting temperature of the double helical DNA [31]. In particular, the mode, strength, detailed energetics and enantioselectivity of the interaction between $[Fe(phen)_2(dppz)]^{2+}$ and ct-DNA have been explored on the basis of the data presented in this study.

Results and Discussion

Absorption spectral features

The absorption of $[Fe(phen)_2(dppz)]^{2+}$ in the visible wavelength region arises from metal-to-ligand charge transfer (MLCT) transitions ($\lambda_{max} = 506$ nm) and from an intraligand (IL) transition in the dppz chromophore ($\lambda_{max} = 375$ and 360 nm) [10 – 11, 18, 21]. A comparison of absorption spectra with those of the parent complex $[Fe(phen)_3]^{2+}$ indicates that the MLCT transitions are only slightly perturbed by the annelation of the phenazine moiety. Similarly, the 375-nm IL transition is hardly perturbed in the metal complex as compared to that of the free ligand dppz ($\lambda_{max} = 378$ nm) [32]. Unlike the absorption spectra of the corresponding complex $[Ru(phen)_2(dppz)]^{2+}$, the MLCT and IL bands of $[Fe(phen)_2(dppz)]^{2+}$ do not overlap with each other.

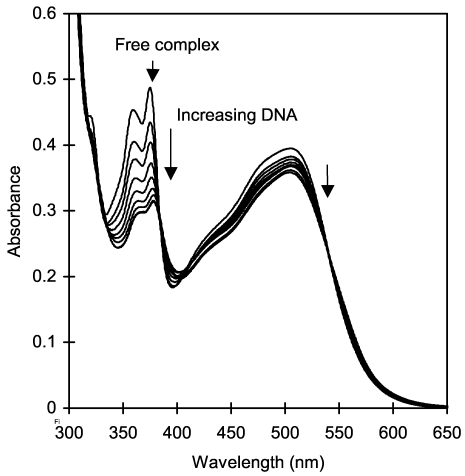

Fig. 2. Absorption spectral changes of 36.5 μM $[Fe(phen)_2(dppz)]^{2+}$ in Tris-HCl buffer at pH 7.2, 50 mM NaCl upon increasing addition of ct-DNA (0.00, 2.89, 5.78, 11.6, 17.3, 26.0, 37.5, 57.6, 86.2 μM).

This feature makes the monitoring and interpretation of DNA binding event of the iron(II) complex by the UV-visible absorption method much easier. The profiles of isotropic absorption of the iron(II) mixed-ligand complex in the presence of ct-DNA are shown in Fig. 2.

The addition of increasing amounts of ct-DNA resulted in the hypochromism and bathochromic shift of the absorption maxima in the UV-visible spectra of $[Fe(phen)_2(dppz)]^{2+}$, of which the phenomenon is very pronounced in the IL band. As can be seen in Fig. 2, a decrease in the absorbance of the IL band is 36% at R ([complex]/[ligand]) = 0.1 and the lowest energy band of $[Fe(phen)_2(dppz)]^{2+}$ ($\lambda_{max} = 360$ nm, $\pi \rightarrow \pi^*$ of dppz ligand) exhibits a bathochromic shift of 8 nm in the presence of ct-DNA, suggesting that the dppz ligand is involved in a strong interaction with ct-DNA. Spectrophotometric titration curves measured between 300 and 650 nm revealed that two isobestic points are present at 384 and 540 nm, indicating that there is an equilibrium state between metal complex and ct-DNA. In the presence of ct-DNA, the MLCT band gives rise to a small blue shift (*ca*. 3 nm) and only a moderate hypochromicity (10% at $R = 0.1$). This suggests that the phen ligands are possibly only involved in an outside binding along the phosphate backbone of DNA. These spectral features are quite similar to those reported for the DNA binding of several tris-chelated and mixed-ligand complexes containing phen and/or dppz. Two different spectral features were observed during the spectrophotometric titration of the mixed-ligand complexes with ct-DNA. The absorbances at 360 and 376 nm decrease linearly with an increase in DNA concentration to $R = 0.5$, while for the mixing ratios smaller than 0.5, they increase with a decrease in mixing ratio and only a slight concomitant change of shape is observed. Indeed, quite similar behavior in the absorption has also been reported for $[Ru(phen)_2(dppz)]^{2+}$ [18], although no reasonable explanation has been presented.

The equilibrium binding constant, K_b, which was obtained by monitoring the variation of absorbance at 375 nm with increasing concentrations of ct-DNA and calculated by eq. (1), is as large as 1.57×10^5 M^{-1} in the medium containing 50 mM NaCl and 5 mM Tris-HCl buffer pH 7.2 at 25 °C. This K_b value is comparable to those reported for the related complexes containing various metal ions such as Os^{II}, Re^{I}, Co^{II} and Ru^{II} ($K_b \cong 10^4 - 10^7$ M^{-1} depending on the ionic strength of the medium). Results of spectroscopic and biochemical studies have suggested that the complexes mentioned above are intercalated into the base pairs of DNA *via* the dppz ligand [10 – 11, 18 – 23]. On the basis of the similarities in structures, spectral profiles, spectrophotometric titration characteristics and equilibrium binding constants between the dppz complexes previously reported and $[Fe(phen)_2(dppz)]^{2+}$, it can be concluded that the DNA binding of the latter complex is also due to an intercalation of dppz as evidenced by the large hypochromicity and moderate red shift at the $\pi \rightarrow \pi^*$ transitions of the dppz ligand. This conclusion is further supported by thermal denaturation experiments described in the following section.

Thermal denaturation

The dissociation of a duplex nucleic acid into two single strands results in significant hyperchromism around 260 nm. The binding of a metal complex to a nucleic acid induces a conformational change of the latter to alter so-called denaturation temperatures depending on the strength and mode of interactions between complex and nucleic acid. In general, groove binding or electrostatic binding along the phosphate backbone of DNA gives rise to only a small change in thermal denaturation temperature, while intercalation leads to a significant rise in thermal denaturation temperature of DNA due to the stabilization of the Watson-Crick base-paired duplex. Therefore, the thermal denaturation experiment of DNA provides a convenient tool for detecting binding and also assessing

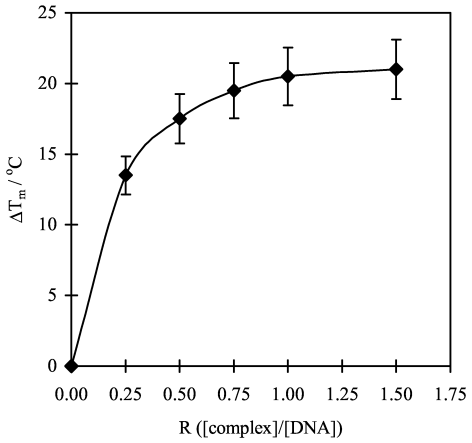

Fig. 3. Profile of denaturation temperature difference (ΔT_m) of ct-DNA as a function of R (=[complex]/[ct-DNA]) ratio in 50 mM NaCl, 5 mM Tris-HCl buffer at pH 7.2.

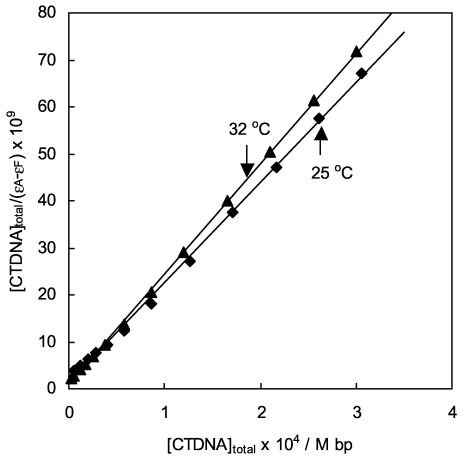

Fig. 4. Typical plots of $[\text{ct-DNA}]_{\text{total}}$ *vs*. $[\text{DNA}]_{\text{total}}$ / $(|\varepsilon_B - \varepsilon_F|)$ for the determination of equilibrium binding constants (K_b) at various temperatures.

relative binding strengths [38]. In the present work, thermal denaturation experiments carried out on ct-DNA in the absence of $[\text{Fe(phen)}_2\text{(dppz)}]^{2+}$ revealed that the thermal denaturation temperature (T_m) of unbound ct-DNA is 74.5 °C under our experimental conditions. As expected, addition of $[\text{Fe(phen)}_2\text{(dppz)}]^{2+}$ to the ct-DNA solution results in a significant rise in the T_m of ct-DNA to varying extents with the increasing ratio of R. The denaturation temperature difference (ΔT_m), *i.e.* the difference in thermal denaturation temperatures between complex-bound and free ct-DNAs is illustrated as a function of R in Fig. 3. The T_m of ct-DNA reaches its maximum value at 95.5 °C when $R = 1.5$ or $\Delta T_m = 21.0$ °C. This ΔT_m value is characteristic of the intercalative binding ($\Delta T_m > 10$ °C) and even larger than those observed for common organic intercalators such as ethidium (13 °C) [33] or some derivatives of porphyrins (≈ 15 °C) [26–28]. A large value of ΔT_m obtained for the thermal denaturation of ct-DNA in the presence of $[\text{Fe(phen)}_2\text{(dppz)}]^{2+}$ is probably contributed in part by the effect of charge of $[\text{Fe(phen)}_2\text{(dppz)}]^{2+}$ (+2) which is larger than the charge of ethidium bromide (+1) [34]. The results of thermal denaturation experiments presented here are consistent with the absorption spectral profiles which demonstrate a significant hypochromicity and a moderate red shift. This indicates that $[\text{Fe(phen)}_2\text{(dppz)}]^{2+}$ binds strongly to ct-DNA mostly in an intercalation mode. Although the maximum value of ΔT_m is obtained at $R = 1.5$, the magnitude of ΔT_m does not differ significantly in the range of $R = 0.5-1.5$. Assuming that the saturated ratio of complex to DNA is reached at $R = 0.5$ or the ratio of [complex] to [DNA] is equal to 1:2, this would indicate that every two base-pairs (four nucleotides) accommodate one molecule of the iron(II) mixed-ligand complex. This consequence is in accord with a neighbor exclusion model of interaction, *i.e.* non-cooperative interaction.

Thermodynamic parameters of the DNA binding

Only few thermodynamic parameters such as free energy, enthalpy and entropy changes upon binding of metal complexes to DNA have been measured, although there have been many reports on the interaction of metal complexes with DNA. In fact, the thermodynamic parameter of DNA-complex formation is essential for a thorough understanding of driving forces of the binding of metal complexes to DNA [35]. To study the thermodynamic parameter of the DNA binding of the iron(II) mixed-ligand complex, the DNA-binding constants of $[\text{Fe(phen)}_2\text{(dppz)}]^{2+}$ have been determined at various temperatures, *i.e.* 11, 18, 25, 32 and 39 °C by spectrophotometric titration and analyzed by using eq. (1). Typical plots of $[\text{DNA}]_{\text{total}}$ *versus* $[\text{DNA}]_{\text{total}}/(|\varepsilon_A - \varepsilon_F|)$ at 25 and 32 °C are shown in Fig. 4. The determination of binding constants at various temperatures provides a good means to indirectly calculate the thermodynamic parameter of the DNA binding by the van't Hoff plot of $1/T$ *versus* lnK_b in the corresponding temperature range (see Fig. 5) [25–26, 28]. Assuming that the enthalpy change (ΔH°) is independent of temperature over the range of employed temperatures, ΔH° of DNA-binding reaction is

Table 1. Comparison of thermodynamic parameters of the DNA binding of iron(II) metal complexes and other binders.

DNA binder	Thermodynamic parameters (kJ mol^{-1})[a]		
	ΔG^o	ΔH^o	$T\Delta S^o$
$[Fe(phen)_2(dppz)]^{2+}$	−29.7	28.1	57.7
$[Fe(phen)_3]^{2+}$[b]	−19.7	30.1	50.2
$[Fe(phen)_2(dip)]^{2+}$[b]	−23.0	32.2	54.4
$[Fe(phen)(dip)_2]^{2+}$[b]	−29.3	−33.1	−3.77
Δ-$[Ru(phen)_2(dppz)]^{2+}$[c]	−37.2	1.05	38.3
Λ-$[Ru(phen)_2(dppz)]^{2+}$[c]	−35.7	12.1	47.7
Ethidium bromide[c]	−32.2	−36.8	−4.60
Daunomycin[c]	−37.7	−43.5	−5.76

[a] In the solution containing 5 mM Tris-HCl buffer at pH 7.2 and 50 mM NaCl; [b] taken from our previous study (ref. 25); [c] obtained from ref. 18 and refs therein. ΔH^o was determined by the colorimetric method and then used to determine ΔS^o at 20 °C.

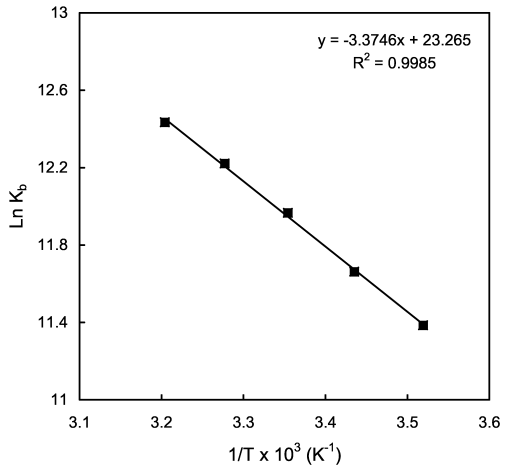

Fig. 5. van't Hoff plot for the binding of $[Fe(phen)_2(dppz)]^{2+}$ to ct-DNA. The slope and intercept are equal to $-\Delta H^o/R$ and $\Delta S^o/R$, respectively.

immediately obtained from the van't Hoff plot (*cf.* Table 1). The striking observation is that the DNA binding of $[Fe(phen)_2(dppz)]^{2+}$ is entropically driven because of the positive enthalpy change. The entropically driven DNA binding of the iron(II) mixed-ligand complex is different from the DNA binding of proven intercalators such as ethidium and daunomycin [20]. However, actinomycin, another proven intercalator, has been reported to bind to DNA with an enthalpy change near zero, indicating that its DNA binding must also be entropically driven [35–37]. Similarly, a positive enthalphy change was also observed for the DNA binding of the corresponding ruthenium(II) complexes Δ- and Λ-$[Ru(phen)_2(dppz)]^{2+}$ which intercalate into the base pairs of DNA [20]. Metal complexes of the type $[M(phen)_2(dppz)]^{2+}$ and actinomycin have a similar structural feature to each other. Both compounds consist of an intercalative chromophore to which bulky constituents are attached. In case of $[M(phen)_2(dppz)]^{2+}$, the bulky constituents are the two phenanthroline wings, while those of actinomycin are cyclic peptides. Upon intercalation of $[M(phen)_2(dppz)]^{2+}$ or actinomycin into the base pairs of DNA, the bulky constituents of both binding ligands, *e.g.* two phenanthroline wings or cyclic peptides should approach the groove of DNA closely. This kind of interaction tends to give similar thermodynamic parameters characterized by positive enthalpy and large positive entropy changes. In contrast, ethidium and daunomycin bear only a small constituent, *i.e.* phenyl group and an amino sugar, respectively. The constituent, therefore, can readily enter the minor groove of DNA, so that the intercalation of either ethidium or daunomycin into the base pairs of DNA needs scarcely energies to compensate. The possible explanation of the entropically driven DNA binding of these types of metal complexes has been discussed in details from the viewpoint of molecular interaction by Haq *et. al.* [20] and by us [25]. In general, the counter ion release, change in hydration and hydrophobic interaction, which are caused by the transfer of the intercalative dppz moiety from the aqueous solvent to the interior of the DNA helix, are the most plausible processes to yield the DNA binding entropically driven. The free energy changes in the DNA binding of $[Fe(phen)_2(dppz)]^{2+}$ are comparable to those reported for the proven intercalators like ethidium bromide and daunomycin as well as to those for the corresponding ruthenium(II) complexes (*cf.* Table 1). These results provide an additional support that the complex $[Fe(phen)_2(dppz)]^{2+}$ undoubtedly interacts with ct-DNA in an intercalation mode.

Enantioselectivity of DNA binding

It has been noted in many reports that a racemic mixture of labile metal complexes, *e.g.* iron(II) and nickel(II) complexes of bpy and phen, undergoes an enantiomeric equilibrium shift (Pfeiffer effect) upon their interaction with optically active compounds like DNA, RNA and protein [25, 38–41]. This yields a certain net excess of the enantiomer preferentially bound to the optically active biopolymer and thus the enantioselective binding is readily monitored in the laboratory by a CD spectropolarimeter. Here, the enantioselectivity of the DNA binding of $[Fe(phen)_2(dppz)]^{2+}$ was studied by examining the CD spectroscopic behav-

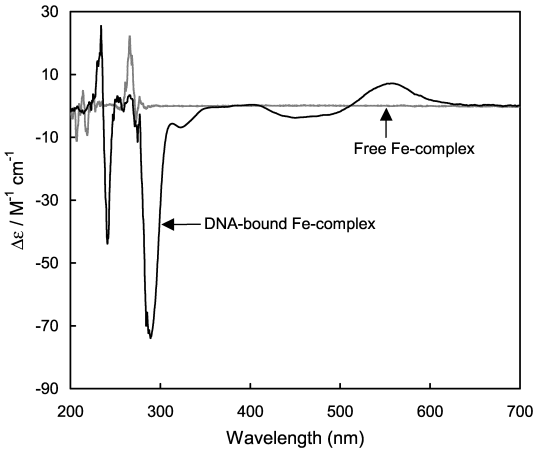

Fig. 6. Circular dichroism (CD) spectra of free and ct-DNA-bound $[Fe(phen)_2(dppz)]^{2+}$ in the UV and visible region of wavelength. The development of CD spectra upon the addition of ct-DNA to the racemic solution of $[Fe(phen)_2(dppz)]^{2+}$ indicates that Pfeiffer effect occurs in the solution.

ior of racemic $[Fe(phen)_2(dppz)]^{2+}$ in the absence and presence of ct-DNA. In fact, it has been well known that a pair of enantiomers of an optically active drug has a different biological activity, although they are structurally similar to each other [29, 30]. The UV-visible CD spectra of racemic $[Fe(phen)_2(dppz)]^{2+}$ were measured at least after 2 h from the addition of a certain amount of ct-DNA and thus after attaining equilibrium. The CD spectra of $[Fe(phen)_2(dppz)]^{2+}$ in the absence and presence of ct-DNA (at $R = 0.1$) are illustrated in Fig. 6. Clearly strong CD was observed in the UV and visible region of wavelength upon addition of ct-DNA to the solution of racemic $[Fe(phen)_2(dppz)]^{2+}$. The development of the strong CD suggests that the change of the 1:1 ratio of metal complex-DNA epimers, *i.e.* an enrichment of one enantiomer, occurs in the solution due to a shift in the enantiomeric equilibrium (Pfeiffer effect). Indeed, no CD signals are observed in the UV and visible region for the free ct-DNA-unbound racemates of the complex. This finding can also be taken as additional independent evidence for the strong interaction of $[Fe(phen)_2(dppz)]^{2+}$ with ct-DNA. Quite similar phenomena have been observed for the DNA binding of iron(II) complexes with bpy and phen [38 – 40] and for that of iron(II) mixed-ligand complexes containing phen and dip (dip = 4,7-dipheny-1,10-phen) [25]. In addition, Taura [41] also observed the same effect as described above when an inversion-labile complex of tris(oxalato)cobaltate(III) was mixed with bovine serum albumin (BSA) in solution. In the previous study [25], we have thoroughly investigated the kinetic aspect of the Pfeiffer effect induced by iron(II) mixed-ligand complexes containing phen and dip and come to the conclusion that the equilibrium shift from Λ- to Δ-enantiomer occurs after the interaction of the iron(II) complex with DNA. This conclusion is deduced from the fact that the CD develops slowly at the same rate as that of the racemization of the iron(II) complex. A careful examination of Fig. 6 has immediately revealed that the CD spectral profiles obtained are the opposite pattern to those of the Λ-enantiomer of iron(II) complexes containing bpy or phen [25, 38 -dd- 40]. In other words, the Δ-enantiomer of iron(II) mixed-ligand complexes is enriched in the solution compared to the Λ-enantiomer. This unequivocally demonstrates that Δ-$[Fe(phen)_2(dppz)]^{2+}$ favorably binds to ct-DNA. The favorable binding of Δ-enantiomers to DNA has been noted in many reports and attributed to the fact that the structure of the Δ-enantiomer precisely fits to the right-handed double helix of ct-DNA.

In addition, the percentage of excess Δ-$[Fe(phen)_2(dppz)]^{2+}$-DNA diastereomer over Λ-$[Fe(phen)_2(dppz)]^{2+}$-DNA may be estimated by comparing the magnitude of the CD spectral signal of DNA-bound $[Fe(phen)_2(dppz)]^{2+}$ in the visible region with that of free enantiomer Λ-$[Fe(phen)_3]^{2+}$. It is expected that the magnitude of the CD spectral signal for each free enantiomer of $[Fe(phen)_2(dppz)]^{2+}$ in the visible region will not differ significantly from that of each free enantiomer of $[Fe(phen)_3]^{2+}$, *e.g.* $\Delta\varepsilon \cong +20$ or -20 (*cf.* Table 2), because the geometrical structure, the absorption spectral pattern at the visible region and the molar absorptivity of the two complexes are quite similar. This approximate comparison is reasonable since our attempt to isolate the free Δ-$[Fe(phen)_2(dppz)]^{2+}$ complex was not successful using the same procedure as used for the isolation of Δ-$[Fe(phen)_3]^{2+}$. Assuming that Δ- and Λ-$[Fe(phen)_2(dppz)]^{2+}$ exhibit a CD spectrum with an identical but opposite sign upon binding to DNA, it is estimated that the percentage of excess Δ-$[Fe(phen)_2(dppz)]^{2+}$-DNA diastereomer over the Λ-$[Fe(phen)_2(dppz)]^{2+}$-DNA must be around 36% in the solution containing 50 mM of NaCl at 25 °C. Using this value we may further calculate the ratio of Δ-$[Fe(phen)_2(dppz)]^{2+}$-DNA to Λ-$[Fe(phen)_2(dppz)]^{2+}$-DNA in the solution (K_{inv}) which is a quantitative measure of the degree of

Table 2. Molar absorptivity (ε) and CD spectral data of the ct-DNA binding of iron(II) complexes[a].

Complex	Molar absorptivity	CD spectral parameters	
	ε ($M^{-1}cm^{-1}$)(λ_{max}(nm))	$\Delta\varepsilon$ ($M^{-1}cm^{-1}$)(λ_{max}(nm))	K_{inv}[b]
free Λ-$[Fe(phen)_3]^{2+}$	11900 (510)	+17 (458)[c]; −21 (542)[c]	1.00
rac-$[Fe(phen)_3]^{2+}$[d]	11900 (510)	−2.0 (464); +3.0 (542)	1.35
rac-$[Fe(phen)_2(dppz)]^{2+}$	10300 (506)	−3.8 (449); +7.2 (556)	2.13

[a] Referred to a solution containing 5 mM Tris-HCl buffer at pH 7.2 and 50 mM NaCl at 25°C; [b] defined as a ratio of Δ-enantiomer to Λ-enantiomer in solution; [c] referred to the free enantiomer. The $\Delta\varepsilon$ values were obtained by extrapolation to zero-time; [d] taken from our previous study (ref. 25).

enantioselective binding of the iron(II) complex to DNA. The calculation results are collected in Table 2 together with those for $[Fe(phen)_3]^{2+}$ [25] as well as molar absorptivity and CD spectral data. It is clearly shown in the Table 2 that substitution of one phen ligand for dppz doubles the enantioselectivity of the Δ-complex towards double-helical DNA. In the separate study, Norden and coworkers [35] have reported a K_{inv} value of 2.0 for the DNA binding of $[Fe(phen)_3]^{2+}$ in the solution containing 10 mM of NaCl and investigated the effect of salt concentration on the K_{inv} value of the DNA binding of $[Fe(bpy)_3]^{2+}$, and found that the increase in salt concentration from 1 mM to 10 mM leads to a decrease in K_{inv} from 1.7 to 1.3 due to the polyelectrolyte effect. In contrast, our K_{inv} value presented here is considerably large although the salt concentration is relatively high in our case. This is probably due to the fact that the dppz complex is intercalated into the base pairs of DNA in which its binding affinity is only slightly influenced by ionic strength in the solution.

Experimental Section

Materials

Dipyrido[3,2-*a*:3',2'-*c*]phenazine hemihydrate (98%) was purchased from Strem Chemicals, New Buryport, MA, USA and 1,10-phenanthroline was obtained from Kanto Chemical Co. Inc. (Tokyo, Japan) and used without further purification. Sodium perchlorate for counter ion in chromatographic process and $Fe(NH_4)_2(SO)_4 \cdot 6H_2O$ were purchased from Kanto Chemical Co. Inc. and Wako Chemicals Industries (Tokyo, Japan), respectively. Acetonitrile, acetone, chloroform (all special grade) and double-distilled water (HPLC grade) were obtained from Katayama Chemical Industries and used as received. Tris(1,10-phenanthroline)iron(II) perchlorate, $[Fe(phen)_3](ClO_4)_2$ was synthesized according to the procedure of Schilt and Taylor [42] and the purity of the complex obtained was confirmed by elemental analysis and UV-visible spectroscopy before use as starting materials for the preparation of bis(1,10-phenanthroline)(dipyrido[3,2-*a*:3',2'-*c*]phenazine)iron(II), $[Fe(phen)_2(dppz)]^{2+}$. Calf thymus DNA (ct-DNA) was obtained from Sigma Chemical Co. (USA) and used as received. The sample of ct-DNA was dialyzed in 50 mM NaCl/5 mM Tris, pH 7.2 for at least 24 h before use for DNA-binding experiments. The solution of ct-DNA (*ca.* 10^{-5} M in base-pair phosphate, bp) in the buffer solution gave a ratio of UV absorbance at 260 an 280 nm, A_{260}/A_{280} of *ca.* 1.9, indicating that the ct-DNA was sufficiently free from protein [43]. The concentration of the nucleic acid solution was determined from the reported molar extinction coefficient of $\varepsilon_{259\,nm} = 13100\ M^{-1}\ cm^{-1}$ and expressed in terms of base-pair equivalents per liter [44].

Measurements

The UV-visible spectra were recorded in solution at a certain temperature on a JASCO V-570 spectrophotometer equipped with a JASCO ETC-505T cell temperature controller using 10-mm quartz cells. The ^1H NMR spectra were taken at 300 MHz with a JNM-LA300 NMR spectrometer. A Yanako L-4000 W pump, a Develosil ODS column (10 μm spherical octadecyl silica, 25 × 3 cm i.d., Nomura Chemical Co., Aichi, Japan) equipped with a Kusano-Kagaku KV-3W loop injector (0.59 cm^3) and a JASCO Uvidec 100-III UV detector were used for semi-preparative HPLC for the purification of iron(II) mixed-ligand complexes. The JASCO PU-980 liquid chromatograph equipped with a Reodyne 7125 (100 μl loop) injector, an Inertsil ODS column (5 μm spherical ODS, 250 mm × 4.6 mm i.d., GL Science Co., Tokyo) and a JASCO MULTI-340 multi-channel detector were used for analytical HPLC.

Synthesis of the iron(II) mixed-ligand complex $[Fe(phen)_2(dppz)](ClO_4)_2$

The complex was synthesized by the ligand substitution in acetone-ethanol (50/50 v/v) medium at 45 °C for 24 h, and the product was purified by semi-preparative HPLC followed by subsequent extraction and evaporation using a rotary evaporator. The procedure was modified from that previously reported for the preparation of iron(II) mixed-ligand complexes containing phen and dip (4,7-diphenyl-1,10-phenanthroline) [45]. The crude product was purified by semi-preparative HPLC using a mobile phase of acetonitrile-water (60/40 v/v) and 0.06 M $NaClO_4$ as a counter anion followed by extraction of the complex into chloroform. The molar absorptivity (ε) of the pure complex was determined by

constructing a graph of concentration *vs.* absorbance in the range of $0.2-5\times10^{-5}$ M. The graph obtained strictly obeys the Beer law ($r \geq 0.999$), suggesting that there is no aggregation of mixed-ligand metal complex in this concentration range. The resulting ε was used for estimating the concentration of the iron(II) mixed-ligand complex in the study of its interaction with ct-DNA.

$[Fe(phen)_2(dppz)](ClO_4)_2$ (wine-red solid, 25% yield): ^{1}H NMR (300 MHz, [D$_6$]-DMSO): $\delta = 9.36$ (dd, 2H, CH*dppz*), 8.81 (m, 4H, CH*phen*), 8.52 (dd, 2H, CH*dppz*), 8.39 (m, 4H, CH*phen*), 8.18 (dd, 2H, CH*dppz*), 7.90 (m, 4H, CH*phen*), 7.82 (dd, 2H, CH*dppz*), 7.75 (m, 2H, CH*dppz*), 7.71 (m, 4H, CH*phen*). – UV-vis (Tris-HCl buffer, pH 7.2, $\mu = 50$ mM, 25 °C): λ_{max} (electronic transition, log ε) = 506 nm (MLCT, 4.01), 375 nm (IL of dppz, 4.13), 360 nm (IL of dppz, 4.09), 320 nm (shoulder, 4.03), 270 nm (IL of phen, 5.02), 226 nm (4.84). – HPLC (Inertsil ODS column; acetonitrile/water: 60:40 (v/v), 0.06 M $NaClO_4$; flow rate: 1 ml/min): $t_R = 5.62$ min, purity 98.7%.

Determination of binding constants

The equilibrium binding constant (K_b) for the interaction of $[Fe(phen)_2(dppz)]^{2+}$ with ct-DNA was determined by spectrophotometric titration. The fixed amount of the iron(II) complex (in the order of 10^{-5} M) in 5 mM Tris buffer pH 7.2 and 50 mM NaCl was titrated at a certain temperature with increasing amounts of DNA stock solutions. The changes in absorbance (hypochromicities) upon addition of DNA were monitored at the maximum wavelengths of 360, 375 and 506 nm and the K_b value of iron(II) mixed-ligand complex to ct-DNA was calculated by eq. (1):

$$\frac{[DNA]_{total}}{|\varepsilon_A - \varepsilon_F|} = \frac{[DNA]_{total}}{|\varepsilon_B - \varepsilon_F|} + \frac{1}{K_b(|\varepsilon_B - \varepsilon_F|)} \qquad (1)$$

where ε_A, ε_F and ε_B correspond to A_{obsd}/[complex], the extinction coefficient of the free iron(II) complex, and that of the iron(II) complex in the fully bound form, respectively. In the plot of $[DNA]_{total}/(|\varepsilon_B - \varepsilon_F|)$ *versus* $[DNA]_{total}$, K_b is given by the ratio of the slope to the intercept. Recent reports [11, 46–47] have demonstrated that this equation provides a useful route to obtain binding constants for the broad range of metal complexes containing phen and its derivatives.

Thermal denaturation experiments

The dissociation (melting) of a polynucleotide strand from a double helical DNA manifests itself as hyperchromicity, *i.e.* an increase in absorbance at a wavelength of 260 nm. Due to the stabilization of the duplex structure, the melting temperature (T_m) of polynucleotide is normally elevated upon addition of iron(II) mixed-ligand complexes. The T_m profiles of ct-DNA in the absence and presence of $[Fe(phen)_2(dppz)]^{2+}$ at ratios of [complex]/[DNA] (R); 0.0, 0.25, 0.5, 0.75, 1.0 and 1.5 were measured on a Jasco V-550 UV/VIS spectrophotometer equipped with a Jasco ETC-505T temperature controller. The temperature was raised at a rate of 1.0 °C/min from 25 to 95 °C with a reading of absorbance taken automatically every 10 s.

Enantioselective binding of iron(II) mixed-ligand complex

The enantioselective binding of the iron(II) mixed-ligand complex was followed by measuring the CD spectra of racemic $[Fe(phen)_2(dppz)]^{2+}$ in the absence and presence of ct-DNA at $R = 0.1$ and after attaining equilibrium with a Jasco J-720 WI spectropolarimeter operating at room temperature (*ca.* 25 °C) using a 1-cm-path quartz cell. The scan region of wavelength was between 200 nm and 700 nm and the result was expressed in molar absorptivity differences of right- and left-circularly polarized lights ($\Delta\varepsilon$, M^{-1} cm^{-1}).

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