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Advances on the interaction of polypyridyl Cr(III) complexes with transporting proteins and its potential relevance in photodynamic therapy

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ABSTRACT

The present study reports a detailed investigation into the interaction of $[Cr(phen)_2(dppz)]^{3+}$ and $[Cr(phen)_3]^{3+}$ with transferrin, the key protein for the transport of Fe^{3+} in blood plasma; its cycle holds promise as an attractive system for strategies of drug targeting to tumor tissues. This can allow us to understand further the role of both complexes as sensitizers in photodynamic therapy (PDT). Chromium(III) complexes, $[Cr(phen)_2(dppz)]^{3+}$ and $[Cr(phen)_3]^{3+}$, (phen = 1,10-phenanthroline and dppz = dipyridophenazine), where dppz is a planar bidentate ligand with an extended π system, have been found to bind strongly with apotransferrin (apoTf) with an intrinsic binding constant, K_b, of $(1.8 \pm 0.3) \times 10^5\ M^{-1}$ and $(1.1 \pm 0.1) \times 10^5\ M^{-1}$ at 299 K, for apoTf–$[Cr(phen)_2(dppz)]^{3+}$ and apoTf–$[Cr(phen)_3]^{3+}$, respectively. The interactions of apoTf with the different Cr(III) complexes were assessed employing UV–visible absorption, fluorescence and circular dichroism spectroscopy. The relative fluorescence intensity of the protein decreased when the increasing concentration of Cr(III) complex was added, suggesting that perturbation around the Trp and Tyr residues took place. The analysis of the thermodynamic parameters ΔG, ΔH, ΔS indicated that the presence of the Cr(III) complex stabilizes the protein with a strong entropic contribution. The binding distances and transfer efficiencies for apoTf–$[Cr(phen)_2(dppz)]^{3+}$ and apoTf–$[Cr(phen)_3]^{3+}$ binding reactions were calculated according to Föster theory of non-radiation energy transfer. All these experimental results suggest that $[Cr(phen)_2(dppz)]^{3+}$ and $[Cr(phen)_3]^{3+}$ bind strongly to apoTf indicating that this protein could act as a carrier of these complexes for further applications in PDT.

1. Introduction

Recently, we reveled that $[Cr(phen)_2(dppz)]^{3+}$ and $[Cr(phen)_3]^{3+}$ (phen = 1,10-phenanthroline and dppz = dipyridophenazine), when associated to DNA, have the ability to induce DNA damage after reaching their excited state and the capability of impairing the survival of irradiated bacteria [1]. These distinctive attributes indicate that $[Cr(phen)_2(dppz)]^{3+}$ and $[Cr(phen)_3]^{3+}$ complexes may act as attractive candidates for DNA photocleavage with potential application in photodynamic therapy (PDT). Due to the activity of these complexes as photosensitizers, their interactions with plasmatic proteins, particularly with serum albumin and transferrin, need to be considered since they constitute the major protein in plasma. Binding to these proteins may lead to an alteration of the biological properties of these complexes; proteins can also provide paths for their transportation.

Hence, to gain a deeper insight into the interactions of photosensitizer Cr(III) complexes with serum proteins, we have recently researched the binding properties of $[Cr(phen)_2(dppz)]^{3+}$ with human and bovine serum albumins [2]. In this work an approach for the binding properties of Cr(III) complexes with transferrin is developed.

Transferrin is a monomeric ~80 kDa glycoprotein, with a single polypeptide chain of about 679 amino acid residues and two metal binding sites. It can be divided into two homologous regions or lobes: the N-terminal domain (residues 1–336) and the C-terminal domain (residues 337–679). Each region shows deep cleft capable of binding a metal ion. Each lobe, in turn, consists of two domains (N_1 and N_2 for N-terminal and C_1 and C_2 for C-terminal respectively) connected by a flexible hinge composed of a series of α-helices, which overlay a central β-sheet backbone. Transferrin is stabilized by 19 intra-chain disulfide bonds and protected by three carbohydrate side chains. The binding site in the N- and C-terminal lobes involves coordination with two tyrosines, one histidine, one asparagine and a carbonate ion, in an overall octahedral environment [3–7]. When the binding sites are empty, the protein is described as apotransferrin (apoTf), as opposed to holotransferrin (holo-Tf) when the sites are occupied. One of the major factors governing the binding and release of iron in transferrin is the pH. Transferrin in human serum is only 30% iron-saturation, and the vacant sites can bind another metal ion [7–9]. In addition to Fe^{3+}, it is known that transferrin is able to bind several other metals, for example, Ti^{4+}, Bi^{3+}, and Ru^{3+}, thus leading to potential usage in diagnosis and therapy [10,11]. On the other hand, the well-known transferrin cycle has kept the attention of several researchers in recent

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years in view of the transportation for metals [12–16], and this cycle holds promise as an attractive system for strategies for the transportation of drug targeting to tumor tissues. Indeed, tumor cells exhibit a large demand for iron for their growth and therefore overexpress transferrin receptors [17–20]. This implies that transferrin–metal ion complex should preserve the good affinity for the transferrin receptor and consequently should interact preferentially with cancer cells. It has been shown that transferrin promotes uptake of Ru(III) complexes into tumor cells [21,22]. Thus, the presence of these properties has motivated us to investigate the interaction of polypyridyl Cr(III) complexes with transferrin since this protein could act as a potential carrier of Cr(III) complexes to tumor cells.

Here, we report a detailed study of the interaction of $[Cr(phen)_2(dppz)]^{3+}$ and $[Cr(phen)_3]^{3+}$ (Fig. 1) with serum transferrin. Cr(III) complexes binding properties with apoTf were assessed employing different spectroscopic techniques. The results from this work imply that the protein transferrin could act as a carrier of Cr(III) complexes, and thus shed light on the applications of such molecules for photodynamic therapy.

2. Materials and methods

2.1. Materials

Chromium(III) complexes $[Cr(phen)_2(dppz)](CF_3SO_3)_3$ and $[Cr(phen)_3](ClO_4)_3$ were synthesized according to a previously reported procedure which was slightly modified [23–26]. Tris–HCl (Tris (hydroxymethyl)aminomethane hydrochloride ($NH_2C(CH_2OH)_3$)) was purchased from Anedra. The stock solutions $[Cr(phen)_2(dppz)]^{3+}$ and $[Cr(phen)_3]^{3+}$ were prepared in TRIS buffer (0.1 M Tris, 0.1 M NaCl, pH 7.4 ± 0.1) and the concentrations were calculated using molar absorptivity values of $\varepsilon_{360nm} = 13{,}900\ M^{-1}\ cm^{-1}$ and $\varepsilon_{354nm} = 4200\ M^{-1}\ cm^{-1}$, respectively. Bovine serum apotransferrin (iron essentially free bovine: minimum 98%, cell culture tested T1428) was purchased from Sigma Chemical Co., and used without further purification. The stock solution apoTf was prepared in TRIS buffer and the concentration was calculated using molar absorptivity value of $\varepsilon_{280nm} = 74{,}400\ M^{-1}\ cm^{-1}$[27]. The solutions Cr(III) complexes and protein used for circular dichroism (CD) experiments were prepared in phosphate buffer (4 mM NaH_2PO_4, 0.1 M NaCl, pH 7.4 ± 0.1). Millipore Milli Q water was used for preparing buffer solutions. All other chemical reagents were of analytical grade.

2.2. Spectroscopic measurements

The UV–visible spectra were recorded on an Agilent 8453 diode array detector spectrophotometer equipped with 1.0 cm quartz cells. Emission spectra were recorded at several temperatures on a Quanta Master QM2 spectrofluorometer from Photon Technology International equipped with a Hamamatsu R928 PMT in a photon counting detector using 1.0 cm quartz cells and thermostat bath, the excitation wavelengths were 280 and 295 nm, the excitation and the emission slit widths were set at 5.0 nm, appropriate blanks corresponding to the buffer were subtracted to correct background fluorescence. The CD spectra were recorded on a JASCO J-810 spectropolarimeter. The experiments were carried out in quartz curettes of 1.0 cm path length. CD data were expressed as the mean residue ellipticity ($[\theta]$).

2.3. Fluorescence study

Fluorescence spectroscopy is an important tool to probe the structure and dynamics of biomacromolecules. The quenching effects of $[Cr(phen)_2(dppz)]^{3+}$ and $[Cr(phen)_3]^{3+}$ on apotransferrin were shown by the well-known Stern–Volmer equation (Eq. (1)) [28];

$$F_0 / F = 1 + k_q \tau_0 [Q] = 1 + K_{sv}[Q] \quad (1)$$

where F_0 and F are the fluorescence intensities in the presence and absence of quencher respectively; [Q] is the concentration of quencher (Cr(III) complexes); k_q is the biomolecular quenching rate constant and τ_0 is the average lifetime of the fluorophore in the absence of quencher with a value of 10^{-8} s [29];and K_{sv} is the Stern–Volmer dynamic quenching constant. The fluorescence intensities used in this study were all corrected for absorption of the exciting light and re-absorption of emitted light (internal filter) using Eq. (2) [30]:

$$F_{cor} = F_{obs} 10^{(-\varepsilon_{exc} l_1 c)} \left(1 - 10^{(-\varepsilon_{exc} l_2 c)}\right) \cdot 10^{(-\varepsilon_{em} l_3 c)} \quad (2)$$

where F_{cor} and F_{obs} are the fluorescence intensities corrected and observed, respectively, ε_{exc} and ε_{em} are the extinction coefficients of the system at excitation and emission wavelengths respectively, and l_1, l_2 and l_3 are parameters that take into account the travel distance of the beam in the quartz cell, 0.4, 0.2 and 0.5 cm, respectively.

2.4. Binding assays

The binding constants were determined by fluorescence intensity of apoTf with $[Cr(phen)_2(dppz)]^{3+}$ and $[Cr(phen)_3]^{3+}$. First, the emission spectrum of protein was recorded using a 2 mL aliquot at a concentration of 1.5×10^{-6} M and subsequently, the spectra of the same solution of the protein were recorded in the presence of increasing concentrations of Cr(III) complex, ranged from 0 up to 10×10^{-6} M, approximately. The volumes of the chromium solution varied from 5 μL up to 780 μL, approximately from a stock solution (4×10^{-5} M). It was not necessary to correct spectra for dilution, because the working solution Cr(III) complexes had the appropriate concentration of protein (1.5×10^{-6} M). After each addition, the solution was incubated for a period of 5 min prior to record the spectrum. The intrinsic binding constants were determined from the decrease of the intensity at 320 nm ($\lambda_{max.}$), with increasing concentrations of Cr(III) complex by using Eq. (3)[2,31]:

$$1 / \Delta F = 1 / \Delta F_{max.} + (1 / \Delta F_{max.} K_b) \times (1 / [Q]), \quad (3)$$

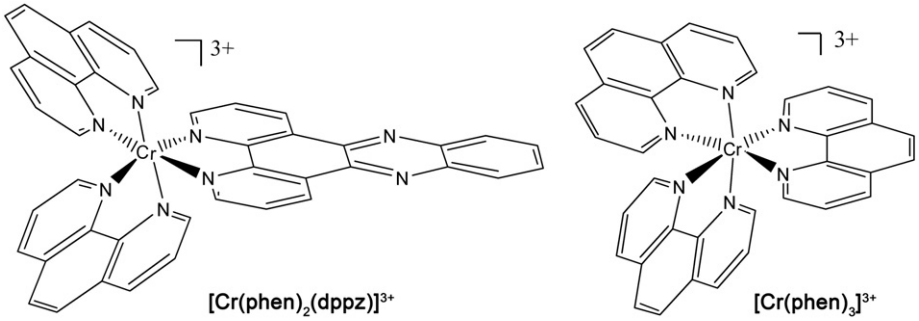

Fig. 1. Molecular structures of the polypyridyl Cr(III) complexes investigated in this study.

where $\Delta F = F_\chi - F_0$ and $\Delta F_{max.} = F_\infty - F_0$, where F_0, F_χ and F_∞ are the fluorescence intensities of protein in the absence of Cr(III) complex, at an intermediate concentration of Cr(III) complex and at the saturation of interaction, respectively; K_b being the binding constant and [Q] the Cr(III) complex concentration. The linearity in the plot of $1/\Delta F$ against $1/[Q]$ confirms a one-to-one interaction between the two components. The binding constants were determined at several temperatures to calculate the thermodynamic parameters for the association processes.

2.5. Resonance energy transfer

In this work, the efficiency of energy transfer was studied according to Förster's non-radiative energy transfer theory where the efficiency of energy transfer, E, is described by the following Eq. (4)[32,33]:

$$E = 1 - F/F_0 = R_0^6 / \left(R_0^6 + r^6\right) \quad (4)$$

where F and F_0 are the fluorescence intensities of apotransferrin (donor) in the presence and absence of Cr(III) complex (acceptor), r is the distance between acceptor and donor, and R_0 is the critical distance when the transfer efficiency is 50%. R_0 is determined from the following Eq. (5):

$$R_0^6 = 8.8 \times 10^{-25}\, k^2\ \eta^{-4}\, \Phi J \quad (5)$$

here, κ^2 is the spatial orientation factor describing the relative orientation in the space of the transition dipoles of the donor and acceptor, η is the refractive index of the medium, Φ is the fluorescence quantum yield of the donor in the absence of the acceptor, and J is the overlap integral of the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor. J is given by Eq. (6):

$$J = \left[\sum F(\lambda)\varepsilon(\lambda)\lambda^4 \Delta\lambda\right] \Big/ \left[\sum F(\lambda)\Delta\lambda\right] \quad (6)$$

where $F(\lambda)$ is the fluorescence intensity of the donor at wavelength λ, and $\varepsilon(\lambda)$ is the molar absorption coefficient of the acceptor at wavelength λ when both spectra are recorded at the same concentration. In this case, $\kappa^2 = 2/3$, $\eta = 1.33$ and $\Phi = 0.13$ [33,34]. From the above relationships, J and E can be easily obtained; therefore, R_0 and r can be further calculated for a molar ratio Cr(III) complex: protein 1:1.

2.6. Circular dichroism study

CD spectra of apoTf ($c_{protein} = 1 \times 10^{-4}$ M in phosphate buffer) and the apoTf–Cr(III) complex at a molar ratio (r_i) of Cr(III) to protein of $r_i = 0.1$ were recorded in the wavelength region 250 to 500 nm. The corresponding absorbance contributions of buffer and free Cr(III) complex solutions were recorded and subtracted with the same instrumental parameters. The CD results were expressed in terms of mean residue ellipticity ($[\theta]$) in °-cm^2 dmol^{-1} according to the following Eq. (7):

$$[\theta] = \text{observed CD (mdeg)} \Big/ \left(10\, C_p n l\right) \quad (7)$$

where C_p is the molar concentration of the protein (mol cm^{-3}), n the number of amino acid residues, and l is the path length (cm) [35].

3. Results and discussion

For macromolecules, fluorescence measurements can provide information of the binding at the molecular level of small molecular substances to proteins, such as the binding mechanism, binding mode, binding constant, intermolecular distances, etc. Thus, the intrinsic fluorescence of proteins can supply significant information regarding their structure and dynamics, and is often considered in the study of protein folding and association reactions [31,36,37]. It should be noted that the intrinsic fluorescence of protein is very sensitive to its micro-environment. When local surrounding of protein is slightly altered, its intrinsic fluorescence becomes significantly weakened, and factors such as protein conformational transition, bimolecular binding, etc., should be responsible for such weakening.

The fluorescence of apoTf arises from the tryptophan (Trp), tyrosine (Tyr) and phenylalanine (Phe) residues. Indeed, the intrinsic fluorescence of apoTf derives almost completely from Trp and Tyr since Phe has a very low quantum yield. This point of view was supported by the experimental observation of Sulkowska et al. [38]. When a small molecule was bound to apoTf, changes of the intrinsic fluorescence intensity of protein were induced by the micro-environment of the Trp/Tyr residue. The participation of tryptophan and tyrosine groups in the interaction between apoTf and $[Cr(phen)_2(dppz)]^{3+}$ or $[Cr(phen)_3]^{3+}$ were assessed on the basis of different excitation wavelengths. At a wavelength of 280 nm, both tryptophanyl and tyrosyl residues in apoTf became excited, whereas the 295 nm wavelength excited only tryptophanyl residues.

3.1. Fluorescence measurements

As mentioned above, fluorescence measurements supply information on the molecular environment in the vicinity of the chromophore molecules. Fig. 2 shows the effect of $[Cr(phen)_2(dppz)]^{3+}$ and $[Cr(phen)_3]^{3+}$ on the fluorescence intensity of the apoTf. When a fixed concentration of protein was titrated with varying amounts of Cr(III) complex ($[Cr(phen)_2(dppz)]^{3+}$ or $[Cr(phen)_3]^{3+}$), a remarkable fluorescence decrease of apoTf was observed, while the emission maximum, $\lambda_{em.}$, remained almost unchanged in both spectra. This indicates that Cr(III) complexes could

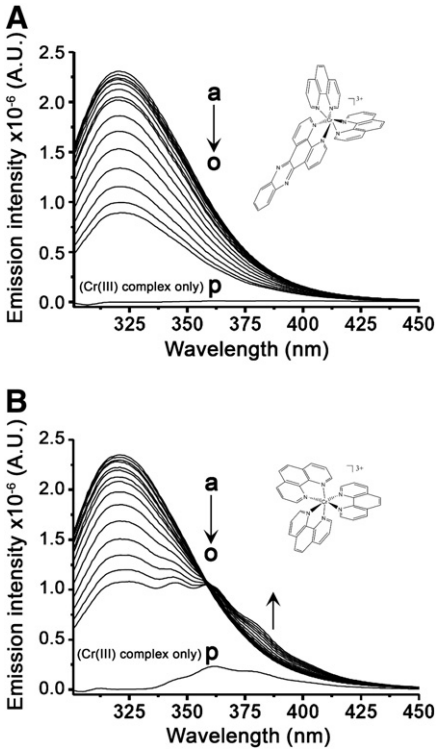

Fig. 2. Fluorescence emission spectra of apoTf in the presence and absence of $[Cr(phen)_2(dppz)]^{3+}$ (A) and $[Cr(phen)_3]^{3+}$ (B).The concentration of protein was fixed as 1.5×10^{-6} M; from a to o the concentrations of Cr(III) complexes were (0, 0.1, 0.2, 0.4, 0.6, 0.8, 1.2, 1.5, 2.3, 3.3, 4.6, 6.1, 7.7, 9.4 and 11) $\times 10^{-6}$ M, respectively; p stands for Cr(III) complexes 10×10^{-6} M; T = 299 K, TRIS buffer pH 7.4; $\lambda_{ex.} = 280$ nm, $\lambda_{em.\ apoTf.} = 320$ nm. Both excitation and emission slits were 5 nm.

bind to apoTf without altering the environment around of the chromophore residues. In addition, Fig. 2A (curves p bottom line) shows the fluorescence spectrum of $[Cr(phen)_2(dppz)]^{3+}$ only, which suggests that the effect of this complex at the excitation wavelength of 280 nm would be negligible; however, as seen in Fig. 2B (curves p bottom line), $[Cr(phen)_3]^{3+}$ displays a slight intrinsic fluorescence without any significant effect on $\lambda_{em.}$. The same behavior was observed in all the range of temperatures used in this work.

The decrease in fluorescence intensity resulted from the diminution of the fluorescence quantum yield, as a consequence of a decrease in the electronic density once the Cr(III) complex molecule entered the hydrophobic cavity of apoTf. The quenching took place when the quencher was sufficiently close to the tryptophanyl and/or tyrosyl residues.

In order to determine whether both residues are involved in the interaction with the different Cr(III) complexes studied, the fluorescence of apoTf excited at 280 and 295 nm in the presence of the different Cr(III) complexes was compared. When the molar ratio (r_i) of Cr(III) to protein was $r_i = 3.7$, the fluorescence of apoTf excited at 280 and 295 nm decreased by 79% and 67%, respectively in the presence of $[Cr(phen)_2(dppz)]^{3+}$ (Fig. 3A) and 58% and 40%, respectively in the presence of $[Cr(phen)_3]^{3+}$ (Fig. 3B). This significant difference between the quenching of apoTf shows that tyrosine takes part in the molecular interactions with both Cr(III) complexes since, at the wavelength 295 nm, only tryptophan is excited.

3.2. Association between polypyridyl Cr(III) complexes and apotransferrin

Fluorescence quenching involves a decrease in the quantum yield of fluorescence from a fluorophore due to a variety of molecular interactions, including excited-state reactions, molecular rearrangements, energy transfer, ground-state complex formation, and collisional quenching. The different mechanisms of the quenching are usually classified either as dynamic quenching or static quenching. Static quenching refers to the formation of a non-fluorescent fluorophore–quencher complex, whereas dynamic quenching refers to the diffusion of the quencher to the fluorophore during the lifetime of the excited state, and upon contact, the fluorophore returns to ground state without emission of a photon [39]. The existence of two modes of quenching for the same fluorophore, static and dynamic, is revealed by a positive curvature in the plot of F_0/F against [Q] (Stern–Volmer plot). A possible quenching mechanism for apoTf by Cr(III) complexes is evidenced from the Stern–Volmer plots (Fig. 4). The Stern–Volmer plots obtained for the quenching of apoTf by $[Cr(phen)_2(dppz)]^{3+}$ and $[Cr(phen)_3]^{3+}$ show positive deviations (Figs. 4A and B). One possible interpretation of this phenomenon was suggested previously [2,38,40] where was interpreted that this type of deviation occurs when quencher interacts with tryptophane and tyrosine residues causing fluorescence quenching; then they change their position within the fluorophore microenvironment making room for the other molecules of the quencher. The same behavior has been observed for the albumin-$[Cr(phen)_2(dppz)]^{3+}$ association process [2].

In order to see the interaction between Cr(III) complexes and apotransferrin, the binding constants, K_b, were determined from the fluorescence intensity data using Eq. (3). The linearity in the plot of $1/(F–F_0)$ against $1/[Q]$ confirms a one-to-one interaction between the two partners [31] (Fig. 5). Table 1 shows the binding constants at three different temperatures (299, 304 and 309 K) for apoTf–$[Cr(phen)_2(dppz)]^{3+}$ system and (295, 299 and 304 K) for apoTf–$[Cr(phen)_3]^{3+}$ system. It was found that the binding constants for both systems were in the order of $10^5\ M^{-1}$, increasing with the rise in temperature and enhancing the stability of protein–Cr(III). The binding constants were used to calculate the thermodynamic parameters. These results are consistent with those found for serum albumin–Cr(III) complex [2]. Moreover, the association constant values of all the systems studied are of the same order of magnitude, which shows that the effect of the

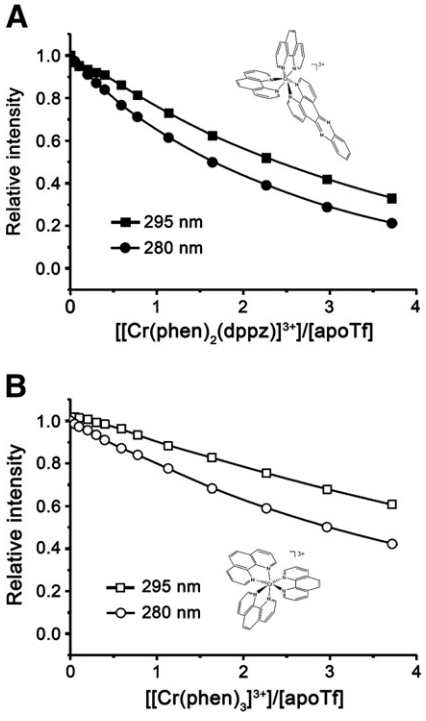

Fig. 3. Plot of relative fluorescence intensity at specific wavelength upon titration with increasing amounts of Cr(III) complex (from 0 to 5.7×10^{-6} M) for apoTf with $[Cr(phen)_2(dppz)]^{3+}$ (A) and $[Cr(phen)_3]^{3+}$ (B). ApoTf–$[Cr(phen)_2(dppz)]^{3+}$ system, $\lambda_{ex.} = 280$ nm (●) and 295 nm (■) T = 304 K and apoTf–$[Cr(phen)_3]^{3+}$ system, $\lambda_{ex.} =$ 280 nm (○) and 295 nm (□) T = 295 K; TRIS buffer pH 7.4.

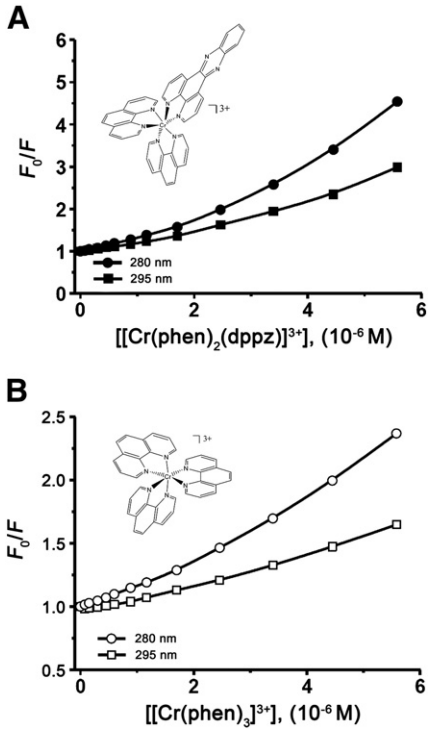

Fig. 4. Stern–Volmer plot of apoTf in the presence of $[Cr(phen)_2(dppz)]^{3+}$ (A) and $[Cr(phen)_3]^{3+}$ (B). ApoTf–$[Cr(phen)_2(dppz)]^{3+}$ system, $\lambda_{ex.} = 280$ nm (●) and 295 nm (■) T = 304 K and apoTf–$[Cr(phen)_3]^{3+}$ system, $\lambda_{ex.} = 280$ nm (○) and 295 nm (□) T = 295 K; TRIS buffer pH 7.4.

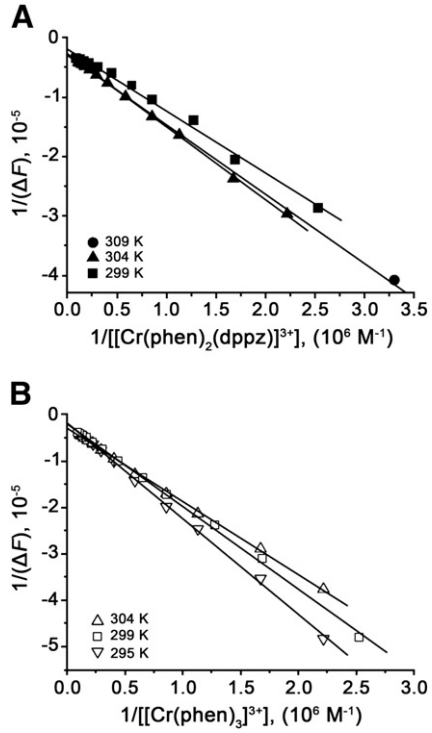

Fig. 5. Plot of $1/\Delta F$ vs $1/[Cr(III)]$ for apoTf–$[Cr(phen)_2(dppz)]^{3+}$ system (A) and apoTf–$[Cr(phen)_3]^{3+}$ system (B); TRIS buffer pH 7.4; $\lambda_{ex.} = 280$ nm at different temperature. The binding constants, K_b, for apoTf–$[Cr(phen)_2(dppz)]^{3+}$ system were (1.8, 2.2 and 2.5) $\times 10^5\ M^{-1}$ at 299, 304 and 309 K, respectively and K_b for apoTf–$[Cr(phen)_3]^{3+}$ system were (0.9, 1.1 and 1.5) $\times 10^5\ M^{-1}$ at 295, 299 and 304 K, respectively.

charge (+3) is not negligible and the small difference found in the value of the constant could be attributed to nature ligand effect (Fig. 1).

3.3. Binding mode and nature of the acting forces

A thermodynamic process was considered to be responsible for the formation of the complex. The thermodynamic parameters were analyzed to further characterize the acting forces in the interaction of apotransferrin with the different Cr(III) complexes studied. The molecular forces contributing to protein interactions with small molecular substance mostly include hydrogen bond, van der Waals force, electrostatic force, hydrophobic interaction force and so on [41]. The thermodynamic parameters, enthalpy (ΔH), entropy (ΔS) and free energy change (ΔG) are fundamental to estimate the binding mode. The thermodynamic parameters (ΔH and ΔS) were evaluated from the slope and y-interception of the Van't Hoff's equation by plotting the values of Ln K_b vs 1/T (Fig. 6). The values of ΔG were further calculated from the values of ΔH and ΔS (Table 2).

Ross and Subramanian [42] have characterized the sign and magnitude of the thermodynamic parameter associated with individual kinds of interaction that may take place in protein association processes, as described below. From the point of view of water

Table 1
Binding parameters obtained from the interaction of different polypyridyl Cr(III) complexes with apotransferrin.

	apoTf–$[Cr(phen)_2(dppz)]^{3+}$		apoTf–$[Cr(phen)_3]^{3+}$	
T (K)	K_b ($\times 10^5\ M^{-1}$)	r	K_b ($\times 10^5\ M^{-1}$)	r
295	–	–	0.9 ± 0.1	0.9995
299	1.8 ± 0.3	0.9967	1.1 ± 0.1	0.9992
304	2.2 ± 0.8	0.9997	1.5 ± 0.8	0.9995
309	2.5 ± 0.1	0.9995	–	–

The parameters were calculated from the data in Fig. 2 using Eq. (3); r is the regression coefficient.

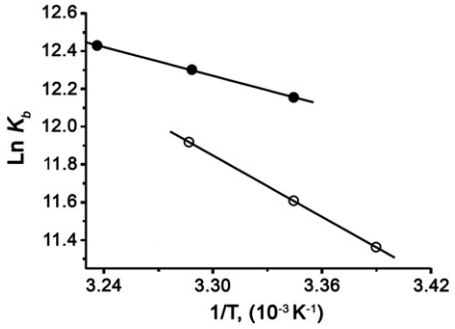

Fig. 6. Van't Hoff plot (Ln K_b vs 1/T) for apoTf–$[Cr(phen)_2(dppz)]^{3+}$ system (●) and apoTf–$[Cr(phen)_3]^{3+}$ system (○).

structure for ligand–protein interaction, the positive ΔS and the positive ΔH are frequently offered as evidence for the hydrophobic interaction, because the water molecules arranged in an orderly fashion around the ligand and protein acquired a more random configuration. Thus, the destruction of the water structure was induced by hydrophobic interactions. Further, interactions between ionic species in aqueous solution are particularly characterized by small ΔH (of either sign) and positive ΔS. The extent of such significant contribution to the free energy from positive ΔS in protein association processes will depend upon the details of specific associations. The negative values of ΔG (Table 2) support the assertion that the binding process is spontaneous. The positive values of ΔS and ΔH observed for the binding processes apoTf–$[Cr(phen)_2(dppz)]^{3+}$ and apoTf–$[Cr(phen)_3]^{3+}$ in the present study indicate that the binding is mainly an entropy-driven process. It can be concluded that the acting are essentially hydrophobic interaction forces, but the electrostatic ones cannot be excluded, in view of the charges of the species involved. These results reveal that the presence of both Cr(III) complexes stabilizes the protein with a strong entropic contribution as found for the process of binding between albumin-$[Cr(phen)_2(dppz)]^{3+}$[2]. These observations are consistent with those made from the quenching of fluorescence (Fig. 2A and B) (see above) where it was proposed that the Cr(III) complex molecule entered the hydrophobic cavity of apoTf, which is next to the specific metal binding sites of the protein, Trp and Tyr residues being involved, among others [43].

3.4. Fluorescence resonance energy transfer (FRET) between apotransferrin and Cr(III) complexes

The importance of the energy transfer in biochemistry lies in the fact that the efficiency of transfer can be used to evaluate the distance between the ligand and the fluorophore in the protein. According to Förster's non-radiative energy transfer theory, the energy transfer will take place under the following conditions: (i) the relative orientation of the donor and acceptor dipoles, (ii) the extent of overlap of fluorescence emission spectrum of the donor with the absorption spectrum of the acceptor, and (iii) the distance between the donor and the acceptor must be lower than 10 nm.

Generally, FRET occurs whenever the emission spectrum of a fluorophore (donor) overlaps the absorbance spectrum of another molecule (acceptor). Here, the donor and acceptor were apoTf and Cr(III) complexes, respectively. The absorption spectrum of $[Cr(phen)_2(dppz)]^{3+}$ and $[Cr(phen)_3]^{3+}$ ($c_{Cr(III)\ complexes} = 1.5 \times 10^{-6}$ M) was recorded in the range of 300–425 nm in TRIS buffer at pH = 7.4. The emission spectrum of apoTf ($c_{protein} = 1.5 \times 10^{-6}$ M) was also recorded under the same conditions, excited at 280 nm. The overlap of the UV absorption spectra of $[Cr(phen)_2(dppz)]^{3+}$ and $[Cr(phen)_3]^{3+}$ with the fluorescence emission spectrum of apoTf is shown in Fig. 7. The distance between the donor and acceptor as well as the extent of spectral overlap determined the extent of energy

Table 2
Thermodynamic parameters of the apotransferrin–polypyridyl Cr(III) complex systems.

T (K)	apoTf–$[Cr(phen)_2(dppz)]^{3+}$			apoTf–$[Cr(phen)_3]^{3+}$		
	ΔG (kJ mol^{-1})	ΔH (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})	ΔG (kJ mol^{-1})	ΔH (kJ mol^{-1})	ΔS (J mol^{-1} K^{-1})
295	–	–	–	-27.9 ± 0.3	44.87 ± 0.02	246.56 ± 0.05
299	-30.2 ± 0.3	21.09 ± 0.05	171.6 ± 0.2	-28.9 ± 0.3	44.87 ± 0.02	246.56 ± 0.05
304	-31.1 ± 0.3	21.09 ± 0.05	171.6 ± 0.2	-30.1 ± 0.3	44.87 ± 0.02	246.56 ± 0.05
309	-31.9 ± 0.3	21.09 ± 0.05	171.6 ± 0.2	–	–	–

Data were calculated with Van't Hoff's equation; the regression coefficients were r = 0.99983 for apoTf–$[Cr(phen)_2(dppz)]^{3+}$ and r = 0.99998 for apoTf–$[Cr(phen)_3]^{3+}$.

transfer. The efficiencies of energy transfer, *E*, were obtained according to Förster's energy transfer theory [32], using Eq. (4). The overlap integral *J* could be evaluated by investigating the spectra seen in Fig. 7. By Eq. (5), the critical distance R_0 could be calculated for the binding process. Table 3 shows the parameters regarding the FRET. Each value of *r* was lower than $2R_0$ and ranged in the efficient distance (2–10 nm) for FRET, which indicated reliability of the results and that the energy transfer from apoTf to $[Cr(phen)_2(dppz)]^{3+}$ and apoTf to $[Cr(phen)_3]^{3+}$ could be likely to occur.

3.5. CD spectroscopy

When drugs bind to a protein, the intermolecular forces responsible for maintaining the secondary and tertiary structures can be altered, resulting in a conformational change in the protein [44]. The circular dichroism (CD) technique is particularly suited to provide information on the specific binding of small substrates to chiral macromolecules [45].

In order to monitor structural changes of the protein produced by the binding of Cr(III) complexes, two regions in the CD spectra of transferrin were analyzed: the aromatic (230–320 nm) and the visible (320–500 nm). It is interesting to study the aromatic region in the case of transferrin since, in the binding, tyrosine residues are directly involved with iron ion, and could provide information on whether this site is involved or not in the binding process of Cr(III) complexes [7,46], since other metal complexes, such as Ru(III) complex, were shown to bind at the same site as that of Fe^{3+}[21]. If this were the case, we would expect to see changes in this region of the spectrum when Cr(III) complex ($[Cr(phen)_2(dppz)]^{3+}$ or $[Cr(phen)_3]^{3+}$) binds to apoTf. Thus, CD spectra of apoTf in the presence and absence of $[Cr(phen)_2(dppz)]^{3+}$ and $[Cr(phen)_3]^{3+}$ were recorded. Both spectra look similar; Fig. 8 shows the spectrum of apoTf in the presence and absence of $[Cr(phen)_2(dppz)]^{3+}$ in the aromatic region where we can note that the intensities of bands (phenylalanine at 255–270 nm, tyrosine at 280 nm and tryptophan at 290 nm) were changed, whereas no CD signals were observed in the visible region, 320–500 nm (Fig. 8 inset). The results indicate that although $[Cr(phen)_2(dppz)]^{3+}$ and $[Cr(phen)_3]^{3+}$ could bind to apoTf, no significant conformation changes are found. In view of the present results, it can be proposed that the binding mode of Cr(III) complexes studied is different from that of Fe(III) and Ru(III) complexes [21]. These observations are consistent with those obtained from fluorescence and thermodynamic experiments, from which it was concluded that the interaction is located in a hydrophobic cavity.

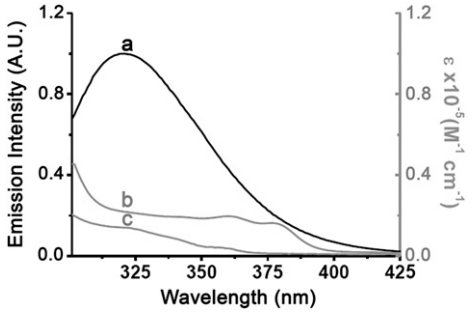

Fig. 7. Overlap of the fluorescence emission spectrum of apoTf (a) with the absorption spectra of $[Cr(phen)_2(dppz)]^{3+}$ (b) and $[Cr(phen)_3]^{3+}$ (c). The concentrations of Cr(III) complexes and protein were 1.5×10^{-6} M; T = 299 K, TRIS buffer pH 7.4; $\lambda_{ex.} = 280$ nm.

Table 3
Parameters regarding the Förster's non-radiative energy transfer theory for the apotransferrin–polypyridyl Cr(III) complex systems.

Parameters	apoTf–$[Cr(phen)_2(dppz)]^{3+}$	apoTf–$[Cr(phen)_3]^{3+}$
J	2.56×10^{-14} cm^3 l mol^{-1}	1.11×10^{-14} cm^3 l mol^{-1}
E	0.125	0.112
r	4.05 nm	3.59 nm
R_0	2.93 nm	2.54 nm

Data were calculated according to Eqs.4 to 6; *J* is the overlap integral of the fluorescence emission spectrum of the donor and the absorption spectrum of the acceptor, *E* is the efficiency of energy transfer, *r* is the distance between acceptor and donor, and R_0 is the critical distance when the transfer efficiency is 50%.

4. Concluding remarks

This paper describes an approach for studying the nature and magnitude of the interactions of apotransferrin with different polypyridyl Cr(III) complexes using fluorescence, absorption and CD techniques. The results reported clearly indicate that $[Cr(phen)_2(dppz)]^{3+}$ and $[Cr(phen)_3]^{3+}$ bind effectively to apotransferrin. The values of association constants of the systems studied are of the same order of magnitude (10^5 M^{-1}), showing that the effect of the charge (+3) is not negligible and that the slight difference in the value of the constant could be attributed to the nature of the ligands. In addition, the sign and magnitude of the thermodynamic parameter of the binding process show that the association between apotransferrin and the different Cr(III) complexes analyzed is spontaneous and largely mediated by hydrophobic as well as electrostatic forces. In addition, and in agreement with the results found in the fluorescence study, CD

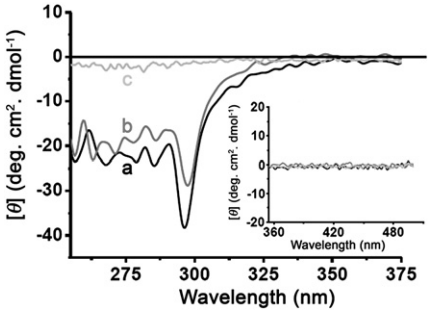

Fig. 8. CD spectra of apoTf (a), apoTf–$[Cr(phen)_2(dppz)]^{3+}$ (b) and $[Cr(phen)_2(dppz)]^{3+}$ (c). The concentrations were $c_{protein} = 1 \times 10^{-4}$ M and $c_{Cr(III)} = 1 \times 10^{-5}$ M; T = 298 K, phosphate buffer pH 7.4. Data were accumulated five times with a 1 nm band width and a 0.5 nm resolution at a scan speed of 100 nm/min.

results indicate that although $[Cr(phen)_2(dppz)]^{3+}$ and $[Cr(phen)_3]^{3+}$ could bind to the protein, no conformation change was observed. This suggests that the binding process would occur in a site different from the specific iron binding sites of the protein, probably in hydrophobic patches near to them, Trp and Tyr residues being involved, among others. Due to the activity of this complex as a photosensitizer, the biological significance of this work is evident since apotransferrin could act as a carrier of these complexes inside tumor cells, and shed light on the potential application of these photosensitizers in PDT for cancer treatment.

Abbreviations

dppz	dipyrido[3,2-a:2′3′-c]-phenazine
phen	1,10-phenanthroline
apoTf	bovine serum apotransferrin
PDT	photodynamic therapy
CD	circular dichroism
Tris	Tris(hydroxymethyl)aminomethane
TRIS	buffer solution (0.1 M Tris, 0.1 M NaCl, pH 7.4 ± 0.1)
FRET	Fluorescence Resonant Energy Transfer

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