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Microcalorimetry and binding studies of DNA upon interaction with [pyridine diamine]$_2$[Co(phenanthroline dicarboxylate)$_2$]

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Abstract

The interaction of calf thymus DNA with [pyridine diamine]$_2$[Co(phenanthroline dicarboxylate)$_2$], [**pyda**]$_2$[**Co**(**phendc**)$_2$], complex has been studied at 10 mM phosphate buffer at 27 °C, pH 7.0 using various techniques including isothermal titration calorimetry (ITC), UV–vis spectrophotometry and potentiometric titration. Sigmoidal absorption profile implies the existence of cooperative interactions between DNA and [pyda]$_2$[Co(phendc)$_2$] that induces a conformational change in DNA structure. Scatchard plots confirm this cooperative interaction. The calorimetric enthalpy curve of [pyda]$_2$[Co(phendc)$_2$] upon interaction with DNA is measured by ITC. The enthalpy of binding (ΔH_{bin}) of [pyda]$_2$[Co(phendc)$_2$] with DNA is also obtained spectrophotometrically by determining of equilibrium binding constants at 27 and 37 °C based on Wyman binding potential and van't Hoff relation. The enthalpy of ionization (ΔH_{ion}) is also obtained by acid–base titration at 27 and 37 °C in 100 mM KCl solution using modified differential van't Hoff relation for estimation of (ΔH_{ion}). The binding data was analyzed in a two-set binding sites model based on the Hill equation. The first phase of the calorimetric enthalpy curve is consistent with a first set of binding sites and further phases are consistent with a second set of binding sites.

Keywords: DNA; [pyda]$_2$[Co(phendc)$_2$]; Enthalpy; Binding analysis; Ionization; Microcalorimetry; Unfolding

1. Introduction

There has been considerable interest in exploring local variations in the structure of DNA along the strand and in developing small molecular probes which, like DNA-binding proteins, may be targeted to particular sites or sequences [1]. Chiral metal complexes have been used in designing spectroscopic probes and photoactivated DNA cleaving agents for DNA [2]. Achiral tris (diphenyl phenanthroline) cobalt (III) has been useful in targeting specific sites in a left-handed conformation in super coiled plasmids and viral DNA [3]. Tris-trimethyl phenanthroline ruthenium (II)($[Ru(TMP)_3]^{2+}$ is seen to bind cooperatively to the A-form polymer under conditions where little binding to B-form DNA is detected. Besides electrostatic interactions, binding of the complex to the polynucleotide may involve hydrophobic interactions of ligands bound against the shallow groove of the helix. The $[Ru(TMP)_3]^{2+}$ binds to B-form DNA via two modes, one intercalative, one surface bound [4]. Intercalative binding through the major groove was characterized by an increase in luminescence lifetime of the complex and preferential

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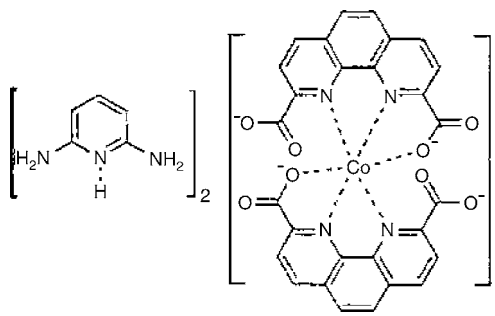
binding to the right-handed helix of Δ-isomer. Phenanthroline complexes might provide the basis for the A-form probe, since a notable feature of the A-helix is the topology of its shallow minor groove, with the major groove deepened, narrowed, and largely inaccessible [5]. The well-known *N*-heterocyclic chelating properties of One to 10 phenanthroline have been widely used in all aspects of coordination chemistry [6] as well as in recent applications to develop biomimetic models of metalloenzymes [7] and to prepare supramolecular [8], self-assembling systems [9], or metal complexes with interesting anti-cancer properties [10]. One to 10 phenanthroline-2,9 dicarboxylic acid(phendc) has been widely employed as an appropriate intermediate in the synthesis of multidentate chelating agents with phenanthroline moieties [11–13]. Spectroscopic studies of the complexation of phendc.H_2 with Eu^{3+} have been reported [14] phendc is a powerful sensitizing ligand for Eu^{3+} ion [15,16] in the measurement of molecules of biological interest, i.e., fibronectin, by time resolved fluorescence spectrophotometry [16,17]. The covalent copper–phenanthroline complex of oligonucleotiedes or nucleic acid-binding molecules in combination with hydrogen peroxide acts as a chemical nuclease to selectively cleave DNA or RNA [18–23]. Hydroxyl radicals or other reactive oxygen species appear to be involved in this cleavage [24]. One to 10 phenanthroline cleaves DNA in the presence of copper and a reducing agent, and with photochemically active para-nitrobenzamide groups on the 4 position, generates clustered lesions or multiply damaged sites (MDS) in lengths of a few base pairs [25–27]. The photochemical and electrochemical properties of copper (I) phenanthroline compounds have also been used to study their interaction with biological systems, in particular DNA intercalation and scission [28]. It could also be an interesting case of the supramolecular and foldamer chemistry [29,30] because of the presence of two bifunctional building blocks, pyda and phendc.H_2O. One to 10 phenanthroline containing anionic Co(II) complexes which have efficient proton-transfer ability [30] are expected to interact strongly with DNA. Structure of the ligand, [pyda$_2$[Co(phendc)$_2$], is shown in Scheme 1. The present study investigates the interaction of a new synthetic complex, [pyda]$_2$[Co(phendc)$_2$], with DNA through enthalpy and binding analysis.



Scheme 1.

2. Materials and methods

2.1. Materials

High molecular weight calf thymus DNA (cat no. 240-13) was obtained from Merck. The compound, [pyda]$_2$ [Co(phendc)$_2$], was synthesized and purified as reported previously [30]. All other materials used were of analytical grade. Calorimetric and spectrophotometric experiments were carried out in 10.0 mM phosphate buffer, pH 7.0 at 27 °C. Potentiometric measurements were performed in 100 mM KCl solution. DNA concentrations were determined with an extinction coefficient of 13200.0 M^{-1} cm^{-1} at 260 nm and expressed in terms of base pair molarity (mM bp) [31]. All solutions were prepared with deionized water (Barnstead Nanopure E.R. = 18.3 MΩ).

3. Methods

3.1. Isothermal titration calorimetry (ITC)

Enthalpy measurements were made at 27 °C with a 2277 thermal activity monitor, (Thermometric, Sweden). The calorimeter was interfaced with a Pentium III computer, using Thermometric Digitam 3 software. The enthalpy of interaction between [pyda]$_2$[Co(phendc)$_2$] and DNA was measured in a ligand concentration range of 0.0–0.2 mM by transferring of 40 μl injections of 0.72 mM [pyda]$_2$[Co(phendc)$_2$] into 1.8 ml of DNA solution (0.028 mM bp). The enthalpy of dilution of [pyda]$_2$[Co(phendc)$_2$] was corrected by measuring the enthalpy change of each injection into buffer solution. The heat released by DNA dilution was negligible.

3.2. Binding measurements

Titration difference spectrophotometry was done with a Camspec double beam spectrophotometer Model M-350. The interaction of DNA with various concentrations of [pyda]$_2$[Co(phendc)$_2$] (0.0–0.15 mM) was studied spectrophotometrically at 260 nm (Fig. 1).

Binding data were obtained from the following relations [32]

$$A = \varepsilon_{\text{free}}[\text{DNA}] + \varepsilon_{\text{bound}}[\text{L-DNA}]$$
$$= \varepsilon_{\text{free}}[\text{DNA}]_{\text{o}} + (\varepsilon_{\text{bound}} - \varepsilon_{\text{free}})[\text{L-DNA}] \quad (1)$$

$$\Delta A_{\infty} = \varepsilon_{\text{bound}}\, b[\text{DNA}]_{\text{o}} \quad (2)$$

where A is the absorbance of the ligand–DNA complex at 260 nm, $\varepsilon_{\text{free}}$ and $\varepsilon_{\text{bound}}$ are the molar extinction coefficients of the free DNA and ligand–DNA complex, and $[\text{DNA}]_{\text{o}}$ and [L-DNA] are concentrations of initial and bound (complexed) DNA. ΔA_{∞} is the observed absorbance change obtained from the titration curve where the absorbance remained unchanged

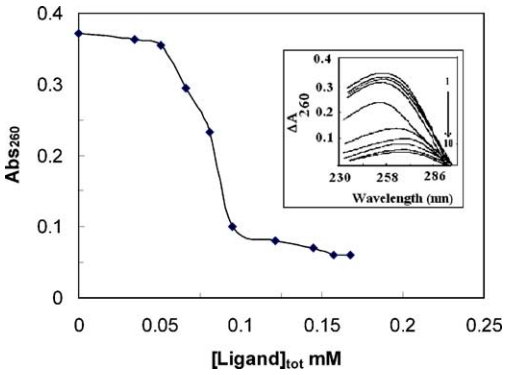


Fig. 1. Typical difference absorbance profile for titration of DNA solution vs. total concentration of $[pyda]_2[Co(phendc)_2]$ in phosphate buffer 10 mM, pH 7.0 at 27 °C. Absorbances are corrected due to dilution of DNA solution with ligand solution. [DNA] = 2.9×10^{-2} mM bp. Inset: Spectrum changes of DNA solution upon interaction with $[pyda]_2[Co(phendc)_2]$.

when excess ligand was added, b the path-length of the cell (1 cm). ε_{bound} is calculable from Eq. (2), the value which is obtained equal to 2642.9 $cm^{-1}\,M^{-1}$ at pH 7.0 and 27 °C, while a value of 13200.0 $M^{-1}\,cm^{-1}$ is used for ε_{free} [31].

Binding data can be obtained by estimating the concentration of bound ligand with Eq. (1). Substitution of $[pyda]_2[Co(phendc)_2]_{bound}$ into Eq. (3) leads to determination of free ligand concentration [33]:

$$[Ligand]_{total} = [Ligand]_{free} + [Ligand]_{bound} \quad (3)$$

Finally ν (average number of moles of ligand bound per DNA base pair mole) is obtained from Eq. (4):

$$\nu = \frac{[Ligand]_{bound}}{[DNA]_o} \quad (4)$$

Also binding data could be analyzed based on the Scatchard plot (Fig. 2, inset) according to Eq. (5) [34]:

$$\frac{\nu}{[Ligand]_{free}} = K(n - \nu) \quad (5)$$

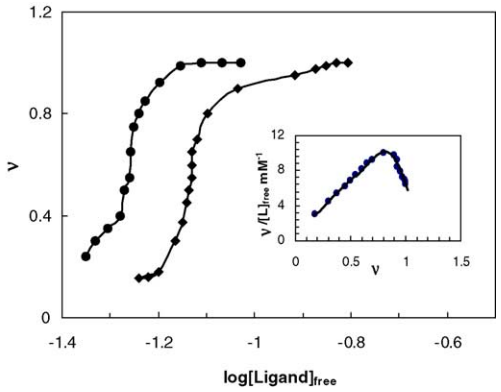


Fig. 2. Binding isotherm for DNA–$[pyda]_2[Co(phendc)_2]$ complex at pH 7.0. Average number of bound ligand (ν) and free concentration of ligand ($[Ligand]_{free}$) are obtained from Eqs. (1) and (3) as described in the text. (●), 27 °C; (■) 37 °C. Inset: Scatchard plot of binding $[pyda]_2[Co(phendc)_2]$ to DNA at 27 °C.

where K and n are the equilibrium binding constant and the number of binding sites, respectively. Binding parameters (ν, and K) for interactions at higher concentrations of $[pyda]_2[Co(phendc)_2]$ (>0.15 mM) are obtained in a similar manner at 260 nm with the Hill equation. Analysis of binding data according to the Hill equation represents two sets of binding sites [35–37] as:

$$\nu = \frac{g_1(K_1[L_1])^{n_{H_1}}}{1 + (K_1[L_1])^{n_{H_1}}} + \frac{g_2(K_2[L_2])^{n_{H_2}}}{1 + (K_2[L_2])^{n_{H_2}}} \quad (6)$$

where g, K, n_H and $[L]$ denote the maximum number of binding sites, binding constant, Hill coefficient, and free concentration of $[pyda]_2[Co(phendc)_2]$ at corresponding sets, respectively. The binding parameters (g, n_H) are obtained by fitting the experimental data to the Hill equation with SigmaPlot software.

3.3. Potentiometric titration

Standard buffers with pH values of 4.0, 7.0 and 9.0 were used for initial scale adjustment of the pH-meter. An automatic and thermostat-controlled microtitrator, Titroline alpha, was used for performing the experiment. It is necessary to carry out a blank experiment on a solution without dissolved DNA for blanking the acid–base equilibria of the solvent. For this purpose, the standardization was achieved as follows: a 4 ml sample of 100 mM KCl solution was added to the titration vessel and equilibrated at 27 °C. To remove carbon dioxide from the mixture, during the experiment nitrogen gas is passed through the stirred solution. Then pH was adjusted to 7.0 with a standard sodium hydroxide solution, and the resultant solution was titrated from pH 7.0 to 2.0 and from pH 7.0 to 12.5 separately [38]. Such standardization was also used after each reversible titration of the DNA sample solution. Thus to 4 ml of an unbuffered solution of 0.50% (w/v) DNA in the absence or presence of $[pyda]_2[Co(phendc)_2]$ at isoionic pH, known amounts of acid or alkali were added followed by potentiometric measurement of the pH of the solution at 27 °C. Addition of acid or alkali is continued until the solution reached pH 2.0 or 12.5, respectively. DNA concentrations were measured spectrophotometrically at pH 7.0 on samples withdrawn from the titration vessel with an extinction coefficient of 13200.0 $cm^{-1}\,M^{-1}$ in terms of base pair molarity of DNA. To estimate the heat of ionization, the same experiments were done at a higher temperature, 37 °C. The heat of ionization of the buffer is negligible and the free and bound ligands are assumed to have the same pK_a value [39]. Potentiometric pH titration data of $[pyda]_2[Co(phendc)_2]$ ligand indicated that the predominant species (~99%) at pH ~ 5–7.5 is the protonated form of ligand (i.e. $[pyda.H]_2[Co(phendc)_2]$) with a pK_a = 7.2 for the pyridine nitrogen. Also protonation pK values for the $-NH_2$ nitrogens of pyda are 2.16 and 2.90 meaning that at neutral pH (our working pH) the pyridinium nitrogen is titratable while the protonated amine groups remain protonated until about pH 10. Hence, the titrated protons of pyda were considered

and correction was made on potentiometric measurements of $[pyda]_2[Co(phendc)_2]$–DNA system. Details of the synthesis, characterization, crystal structure and solution studies of the complex are discussed previously [30]. After obtaining the potentiometric titration curves profiles of r (average number of proton bound by, or removed from the macromolecule) are obtained as the difference between the moles of strong acid or base added to a solution containing one mole of macromolecule to bring it from its initial pH to the final value, and the number of moles of strong acid or base added to the solvent to give the same pH change with other conditions (ionic strength, temperature, volume) being the same [40,41]. Details of the method and calculations are described previously [38]. Titration curves involved the mean ± S.E. of the mean (S.E.M) for three titration experiments.

4. Results and discussion

4.1. Binding data

The difference absorption spectra of the DNA–$[pyda]_2[Co(phendc)_2]$ complex are shown in Fig. 1 inset. Fig. 1 depicts the titration difference absorbance curve at 260 nm for DNA upon interaction with $[pyda]_2[Co(phendc)_2]$.

Fig. 2 shows the binding isotherm curve as a plot of ν (the average number of $[pyda]_2[Co(phendc)_2]$ moles bound to 1 mol of DNA base pairs) against the logarithm of free ligand concentration. The inset of Fig. 2 shows the Scatchard plot, which shows the cooperative manner of binding and existence of two sets of binding sites.

The binding parameters (g and K) according to the Hill equation are obtained as:

$$g_1 = 0.60, \quad g_2 = 1.0, \quad \log K_1 = 15.238,$$

$$\log K_2 = 6.386$$

which show a strong binding in the first set of sites.

ΔG° values for the interaction can be calculated using the Wyman binding potential as described previously [42]. The binding potential and subsequently the apparent equilibrium binding constant (K_{app}), ΔG° and $\Delta G^\circ{}_\nu$ were calculated from the area under the binding isotherm curve (Fig. 2). Fig. 3 depicts the variation of $\Delta G^\circ{}_\nu$ ($\Delta G^\circ/\nu$) (standard Gibbs free energy changes corresponding to the average number of bound ligand) as a function of ν. Fig. 4 shows the binding of ligand is a spontaneous process accompanied by an endothermic binding enthalpy. The figure confirms the electrostatic interaction up to $\nu \sim 0.6$.

4.2. Enthalpy curves

An enthalpy analysis is essential to resolve the probable conformational change of DNA upon interaction with $[pyda]_2[Co(phendc)_2]$. The total enthalpy change associated with interaction of DNA and the ligand can be measured by

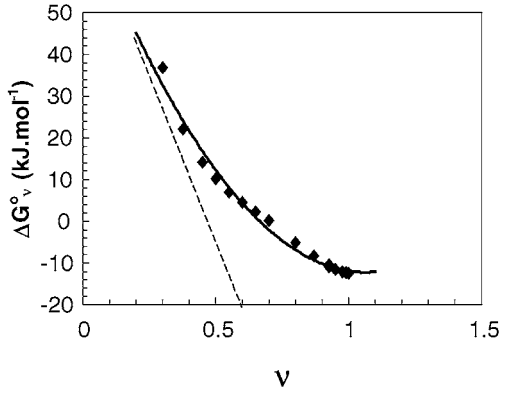


Fig. 3. Variation of $\Delta G^\circ{}_\nu$ ($\Delta G^\circ/\nu$) as a function of ν at pH 7.0 and temperature of 27 °C.

isothermal titration calorimetry. The interaction includes: (a) binding of $[pyda]_2[Co(phendc)_2]$ to DNA; (b) protonation and/or deprotonation of titratable groups in the interaction; and (c) induced conformational changes of DNA structure by binding of the ligand. Thus for the interaction of DNA with $[pyda.H]_2[Co(phendc)_2]$, the calorimetric enthalpy (ΔH_{cal}) consists of contributions from enthalpy of binding (ΔH_{bin}), enthalpy of conformational change (ΔH_{con}) and enthalpy of ionization (ΔH_{ion}). Hence, we can write the equation as follows [38,43]:

$$\Delta H_{cal} = \Delta H_{bin} + \Delta H_{con} + \Delta H_{ion} \tag{7}$$

Fig. 4 shows the enthalpy of binding (ΔH_{bin}) obtained from the temperature dependence of equilibrium binding constants with the van't Hoff relation [44,45] versus ligand concentration.

$$\Delta H_{bin} = \frac{-R \times d(\ln K_{app})}{d(1/T)} \tag{8}$$

Fig. 4 shows decreasing endothermicity of DNA–$[pyda]_2[Co(phendc)_2]$ interaction up to saturation of DNA with ligand.

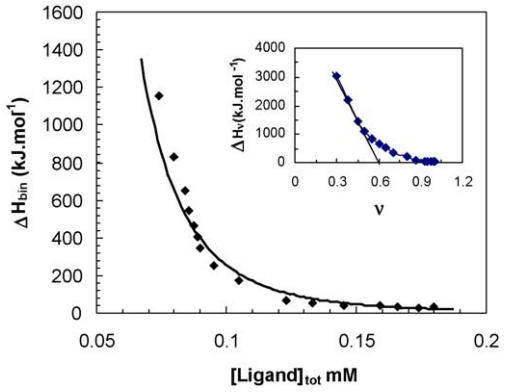


Fig. 4. Variation of enthalpy of binding (ΔH_{bin}) against concentration of $[pyda]_2[Co(phendc)_2]$ at pH 7.0. Inset: $\Delta H_{(bin)\nu}$ ($\Delta H_{bin}/\nu$) as a function of ν.

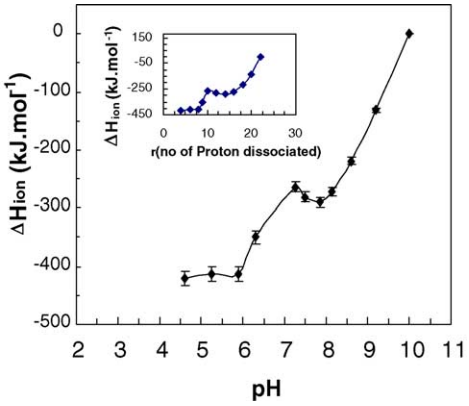


Fig. 5. Ionization enthalpy (ΔH_{ion}) for titratable groups of DNA as a function of pH in 100 mM KCl solution. Enthalpy values are calculated from titration curves at 27 and 37 °C using Eq. (9). Inset: Variation of ΔH_{ion} vs. the average number of dissociated protons, r, through titration experiment in the presence of 0.15 mM of $[pyda]_2[Co(phendc)_2]$.

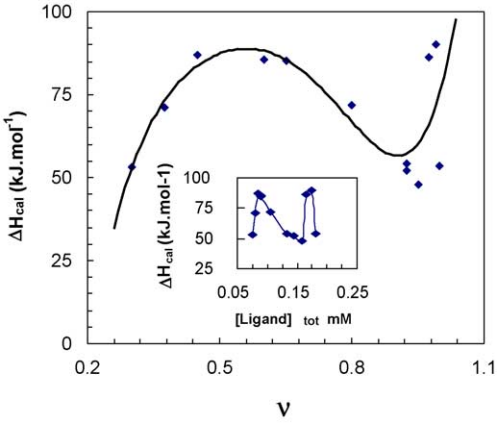


Fig. 6. Variation of calorimetric enthalpy (ΔH_{cal}) as a function of ν at pH 7.0 and 27 °C. Inset: ΔH_{cal} as a function of $[pyda]_2[Co(phendc)_2]$ concentration at pH 7.0 and 27 °C.

Fig. 4 inset represents the changes of ΔH_ν ($\Delta H/\nu$) as a function of ν. An electrostatic interaction up to $\nu \sim 0.6$ is in agreement with the consequence of Fig. 3.

Due to strong electrostatic effects in the binding of such ligands to DNA, the pK_a may shift and a change of titratable groups becomes significant. Therefore we can use a modified van't Hoff relation for estimation of ionization enthalpy (ΔH_{ion}) as follows [46]:

$$\Delta H_{ion} = 2.303R\left[\frac{\delta pH}{\delta(1/T)}\right] \quad (9)$$

Fig. 5 shows the dependence of apparent enthalpy of various titratable groups of DNA on pH in the absence and presence of 0.15 mM of $[pyda]_2[Co(phendc)_2]$ (a concentration of ligand corresponding to maximum binding and saturation of DNA according to Fig. 1). This enthalpy is obtained from the temperature dependence of pH for each solution at a fixed amount of acid or alkali. The figure indicates a less exothermic trend up to pH 10 which corresponds to an enthalpy change of about 421 kJ mol^{-1}.

Fig. 5 inset represents the dependence of ionization enthalpy (ΔH_{ion}) against r value (average number of protons attached or dissociated corresponding to the pH changes). The figure shows that in the presence of 0.15 mM ligand, a constant heat trend is observable up to pH 6, so that the exothermic process is accompanied with titration of 8 titratable groups and the second set of binding sites involves titration of 12 titratable groups of DNA up to pH 10 which evolves 263 kJ mol^{-1} of heat. Therefore, DNA–ligand binding and unfolding of DNA exposes or changes the pK_a values of some titratable groups in pH range of 6.0–10.0 (about 12 titratable groups). Regarding the pK_a values of phosphates (1.5 and 6.5), purine and pyrimidine nitrogens (9.0) and hydroxyl groups of sugars (9.5–10.0), we suggest that in the presence of ligand in the range of pH 3.0–6.0 ($r = 8.0$) binding took place on phosphates. In the range of 6.5–10.0 ($r = 12$), nitrogen of bases and the hydroxyl groups of sugar may participate in binding [46]. The Hill analysis suggests that electrostatic interactions in the first set of binding sites involve highly cooperative interactions of phosphate groups of DNA with $[pyda]_2[Co(phendc)_2]$, while responsible groups for the interaction in the second set could be mostly base and/or hydroxyl groups of DNA.

Fig. 6 (inset) shows the variation of calorimetric enthalpy (ΔH_{cal}) as a function of ligand concentration. ΔH_{cal} is a measure of overall interaction enthalpy between DNA and $[pyda]_2[Co(phendc)_2]$ (at pH 7.0 of phosphate buffer 10 mM and 27 °C). Fig. 6 represents the variation of ΔH_{cal} as a function of ν. As the figure shows, an endothermic stage is observed up to $\nu \sim 0.6$ (corresponding to an enthalpy value of about 85 kJ mol^{-1}) which is in good agreement with the result of binding data analysis at the first set of binding sites ($g_1 = 0.6$). At such condition, the binding process is predominantly endothermic.

Endothermic binding probably occurs on phosphate groups, corresponding to the first set of binding sites and involves occupation of 60% (0.6/1.0) of total binding sites. This may be attributed to binding of the cation of the complex (i.e. protonated pyridine diamine of the pyda cation), which has a high affinity for phosphate groups. Its protonated amine groups have a pK_a value about 10 and hence will be positive up to pH 10. The pyridinium nitrogen has a pK_a value about 7 and can also play a role in the electrostatic interaction. The ionization enthalpy may be the predominant enthalpy change corresponding to the deprotonation of 45% of total titratable groups (mostly phosphate groups). In the second set, binding continues (up to $\nu = 0.95$) through an exothermic trend which may arise from ionization of newly exposed titratable groups (N1 adenine and N3 cytosine). Finally, the process proceeds and completes with a more endothermic phase (up to $\nu = 1.0$), involving the binding of the large anion, [Cobalt(II)(phenanthroline dicarboxylate)$_2$], on nitrogen bases and/or sugar hydroxyl groups of DNA. Subsequently, unfolding of DNA structure can occur probably via hydrophobic interactions between the hydrophobic part of the

ligand and the hydrophobic pocket of guanine and hydrogen bonding and electrostatic interaction between $–COO^-$ of the ligand and –NH and $–NH_2$ groups of DNA bases. A preferential binding between –NH groups of DNA (N7 guanine and adenine, N3 cytosine and thymine) and the Co(II) ion of the ligand can take place because of higher affinity of these nitrogens for the metal ion in comparison to carboxylates of $[pyda]_2[Co(phendc)_2]$. The observation of hypochromicity and red shifts in UV–vis spectra of the interaction as indicated in Fig. 1 inset reinforces this probability [32]. Since nitrogen bases of DNA can be protonated and carry a positive charge at neutral pH, the binding of this macrocyclic anion may introduce a source of hydrophobic interaction as shown by the positive enthalpy changes shown in Fig. 6.

By substituting the values of enthalpies in Eq. (7), ΔH_{con} can be obtained at given conditions. The values of enthalpies at pH 7.0 and [Ligand] = 0.15 mM are equal to $\Delta H_{cal} = 48.05\,kJ\,mol^{-1}$ (Fig. 6 inset); $\Delta H_{ion} = 263.50\,kJ\,mol^{-1}$ (Fig. 5 inset); $\Delta H_{bin} = 40.38\,kJ\,mol^{-1}$ (Fig. 4).

By inserting these values into Eq. (7), ΔH_{con} equals to $271.17\,kJ\,mol^{-1}$. This endothermic conformational enthalpy change confirms the existence of an unfolding process [47]. The endothermic process (reduction of exothermicity) occurs by hydrophobic bonding [48–50]. During the unfolding, exposing of masked titratable groups to the solvent and the subsequent titration of them generates the required energy for unfolding of DNA structure, (see Fig. 5).

5. Conclusion

The results indicate two different behaviors for binding of $[pyda.H]_2[Co(phendc)_2]$ to DNA. An endothermic electrostatic binding process on phosphate groups takes place at low ligand concentrations. The binding process continues through an exothermic trend due to ionization of newly exposed titratable groups of DNA. Finally the process completes by an endothermic reaction change which involves the binding of the large anion, [Cobalt(II)(phenanthroline dicarboxylate)$_2$] to DNA. Consequent unfolding of the DNA structure occurs via hydrophobic interactions. Hence, the enthalpy of the conformational change (ΔH_{con}) of DNA caused by $[pyda.H]_2[Co(phendc)_2]$ binding was determined by resolving the contributions of calorimetric enthalpy, binding enthalpy and ionization enthalpy. The enthalpy of the conformational change (ΔH_{con}) for DNA in the presence of $[pyda.H]_2[Co(phendc)_2]$ (0.15 mM) is equal to $271.17\,kJ\,mol^{-1}$ ($\sim 0.411\,kJ\,g^{-1}$) indicating the partial unfolding of DNA.

The most probable candidates for binding sites in the first set are phosphate groups with pK_a values of 1.5 and 6.0. The partial unfolding of the DNA occurs at higher concentrations of $[pyda]_2[Co(phendc)_2]$ and is accompanied by titration of newly exposed groups. These groups are nitrogen bases (pK_a values about 9) and/or hydroxyls of sugars (pK_a values about 9.5–10) and binding occurs through an endothermic process involving interaction with the base groups.

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