4-Picoline-2,2′:6′,2″-terpyridine-platinum(II) – a potent intercalator of DNA

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Abstract 4-Picoline-2,2′:6′,2″-terpyridine-platinum(II) is shown in a ligation assay to unwind and so intercalate into DNA. Circular dichroism is used to determine an equilibrium binding constant of approximately 2×10^7 M^{-1} for the most stable binding mode of 4-picoline-2,2′:6′,2″-terpyridine-platinum(II) to poly[d(A–T)$_2$] with a site size of about 4 base pairs, and about 1×10^6 M^{-1} for a second binding mode with a site size of about 2 base pairs. Fluorescence spectroscopy provides further evidence for the strong equilibrium binding constant of 4-picoline-2,2′:6′,2″-terpyridine-platinum(II) in that it displaces ethidium bromide bound to DNA. The double positive charge on 4-picoline-2,2′:6′,2″-terpyridine-platinum(II), together with the intercalative binding mode is probably responsible for the large binding constant.

Key words: DNA ligation; Fluorescence spectroscopy; Circular dichroism

1. Introduction

2,2′:6′,2″-Terpyridine-platinum(II) complexes were first reported to bind to ds DNA by intercalation over twenty years ago, the duplex unwinding angle being comparable to that found with ethidium bromide [1]. An investigation of the binding of hydroxyethanethiol-2,2′:6′,2″-terpyridine-platinum(II) **1** (R = SCH_2CH_2OH) with calf-thymus (ct)-DNA by fiber X-ray diffraction techniques showed the platinum ions in the complex to be distributed along the helix axis in accord with the nearest neighbour exclusion model [2]. The fourth ligand to Pt in all the complexes reported were either thiolate or chloride ions so that the complex bore only a single positive charge. The binding association constants with ct-DNA were between 0.23 and 2×10^5 M^{-1}, that of the hydroxyethanethiol complex **1** (R = SCH_2CH_2OH) being 0.83×10^5 M^{-1}. The chloro-platinum complex **1** (R = Cl) had essentially the same binding constant but was shown slowly to form a covalent link to DNA [3]. Recently the hydroxy-platinum complex **1** (R = OH) has been shown to intercalate into DNA with a binding constant of approximately 7×10^4 M^{-1} and to slowly form a covalent bond with the DNA [4].

In the expectation that a 2,2′:6′,2″-terpyridine-platinum(II) complex which retained a double positive charge would bind more effectively to DNA, 4-picoline-2,2′:6′,2″-terpyridine-platinum(II) **2**, was prepared and its interaction with DNA investigated. As preliminary experiments with ct-DNA indicated some degree of sequence variation in the binding, we performed detailed binding studies using circular dichroism (CD) and fluorescence spectroscopy with poly[d(A–T)$_2$], so that any binding changes observed could not be due to sequence variations.

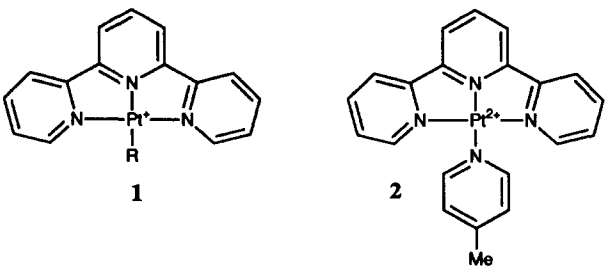
2. Materials and methods

2.1. The ligation assay

Unwinding was detected using an electrophoretic assay and plasmid DNA (5.3 kbp) linearized with *Hin*dIII [5]. 500 ng linear DNA was ligated in 250 μl containing 1 mM ATP, 10 mM $MgCl_2$, 50 mM Tris-HCl (pH7.5), 1 unit T4 DNA ligase (Boehringer) and various concentrations of test compound. [Compounds were dissolved in dimethylsulphoxide as stock concentrates; Fig. 1, lane 20 indicates that this solvent had no effect on electrophoretic mobility.] After incubation on ice overnight, DNA was precipitated with ethanol to remove the test compound, redissolved in a sample buffer containing 1% sodium dodecyl sulphate and then subjected to electrophoresis in a 0.8% agarose gel containing 40 mM Tris, 2 mM EDTA and 20 mM sodium acetate (pH 8.3) at 4°C; finally, the gel was stained with ethidium and photographed [6,7].

2.2. Spectra

Normal absorbance spectra were measured on a Cary 3 spectrometer. Infra-red spectra were recorded on an internally referenced Perkin-Elmer 1750 FT-IR spectrometer. Only selected absorbances (υ_{max}) are quoted (vs = very strong, s = strong, m = medium, w = weak, br = broad absorbances). Mass spectra were recorded on a V.G. Biotech Bio-Q spectrometer [electrospray ionisation (ESI)]. Values are quoted in Daltons per unit charge (*m/z*). Melting points (m.p.) were recorded on a Kofler block apparatus and are uncorrected. Proton magnetic resonance spectra (^{1}H NMR) were recorded at 500 MHz on a Bruker AM500 spectrometer or at 200 MHz on a Varian Gemini spectrometer. ^{1}H NMR kinetic experiments were performed at 250 MHz using a Bruker AM250 spectrometer. Spectra recorded in D_2O are referenced to internal sodium 3-(trimethylsilyl)-tetradeutero-propionate and chemical shifts (δ_H) are in parts per million (ppm). Coupling constants (*J*) are recorded in Hertz to one decimal place (s = singlet, d = doublet, t = triplet, m = multiplet).



Scheme 1.

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2.3. Synthesis of 4-picoline-2,2′:6′:2″-terpyridine-platinum(II) tetrafluoroborate **2**

A solution of silver tetrafluoroborate (0.20 g, 1.03 mmol, excess) in methanol (5.0 cm^3) was added to a solution of [Pt(terpy)Cl]Cl·$2H_2O$ (0.100 g, 0.187 mmol, Aldrich) **1** (R = Cl) in methanol (20.0 cm^3). After stirring for 10 min. 4-picoline (0.0255 cm^3, 0.262 mmol) was added and the resulting mixture heated to reflux, with light excluded, for 10 h. in order to coagulate the silver chloride which was filtered off while the solution was still hot. On cooling the filtrate yellow crystals formed which was filtered off and washed well with cold methanol. Recrystallisation from methanol afforded *4-picoline-2,2′:6′:2″*-terpyridine-platinum(II) tetrafluoroborate (35.1 mg) as bright yellow needles (m.p. > 220°C); υ_{max} (KBr disc) 3387br s, 3083br m, 3032br m, 1606s, 1477m, 1453s, 1400m, 1318m, 1056br vs, 776vs and 722m cm^{-1}; $\lambda_{max}(\varepsilon)$ (H_2O) 240 (28800), 269 (19000), 324 (8700) and 339 (15000) nm; δ_H (500MHz, D_2O) 2.59 (3H, s, -C$\underline{H}_3$), 7.65 (2H, m, 2 × C(5)$\underline{H}$), 7.70 (2H, d, J = 6.3 Hz, 2 × C(8)$\underline{H}$), 7.77 (2H, d, J = 5.6 Hz, 2 × C(6)$\underline{H}$), 8.34–8.37 (4H, m, 2 × C(4)$\underline{H}$ and 2 × C(3)$\underline{H}$), 8.36 (2H, d, J = 9.0 Hz, 2 × C(3′)$\underline{H}$), 8.47 (1H, t, J = 8.3 Hz, C(4′)$\underline{H}$), 8.79 (2H, d, J = 6.6 Hz, 2 × C(7)$\underline{H}$); *m/z* (ESI) 260.5 ([4-picoline-terpyridine-Pt(II)]$^+$, 100%).

2.4. Measurement of concentrations

Calf thymus DNA (ct-DNA) was purchased from Sigma Chemicals Co. Limited and poly[d(A-T)]$_2$ (AT-DNA) from Pharmacia P-L Biochemicals. They were used without further purification. Concentrations were determined spectroscopically using ε_{257} = 6600 for ct-DNA, ε_{260} = 6600 for AT-DNA, ε_{249} = 28,800 for **1** (R = Cl) and ε_{240} = 28,800 for **2**.

2.5. Thermal denaturing studies

Thermal denaturing studies were carried out on the Pt complexes **1** (R = Cl) and **2** with ct-DNA in a Cary 3 spectrometer fitted with a thermostated cell holder. The temperature was increased at a rate of 0.33°C per minute, allowing the system to stabilise for 15 min before taking the first reading. A signal averaging time of 2 s was used. Systems studied were 400 μM ct-DNA with 0 μM or 20 μM Pt complexes **1** (R = Cl) and **2**.

The increase in absorbance at 270 nm, corresponding to the transition from ds to ss DNA was used to monitor the thermal denaturation of ct-DNA in the absence and presence of the Pt complexes **1** (R = Cl) and **2** (Fig. 3). The melting temperature, T_m, is defined as the mid-point of the transition, and is normally calculated by averaging the minimum and maximum absorbance, and interpolating to the mid-point. An alternative, more accurate method is to take T_m as the point of inflexion, found from the derivative of the melting curve. The advantage of this method is that it is much easier to identify when more than one transition occurs.

2.6. Circular dichroism (CD) studies

CD measurements were performed on a Jasco J720 spectropolarimeter. The spectra were recorded for 500 μM AT-DNA at constant 10 mM aqueous NaCl concentration with Pt complex concentrations increasing from 0 to 125 μM.

The main purpose of the CD studies was to determine the equilibrium binding constant, K, using the induced spectroscopy method (ISM)[8]. ISM calculations require the induced CD (ICD) at a given wavelength for a titration series where the DNA concentration is held constant and the ligand concentration is varied (or conversely), to be proportional to the concentration of bound ligand: $c_b = \alpha\rho$ where c_b is the bound concentration and ρ is the ICD. In such a case

$$\frac{\delta c_i}{\delta \rho_i} = \left(\frac{d}{\alpha}\right)\left[\frac{\delta(c_i/\rho_i)}{\delta \rho_i}\right] + \alpha$$

where c_i is the ith ligand concentration, ρ_i is the ith ICD, $\delta c_i = c_i - c_k$ etc., and d = concentration of binding sites on the DNA. If the binding regime is not uniform a random plot results. Once α and d are known, the equilibrium binding constant may be determined, since

$$K = \frac{c_b}{S_f \cdot c_f} = \frac{\alpha\rho}{(d-\alpha\rho)(c_{tot} - \alpha\rho)}$$

where S_f is concentration of free sites, c_b is concentration of bound ligand, and c_{tot} is the total ligand concentration.

2.7. Fluorescence studies

Fluorescence measurements were performed on a Perkin-Elmer LS-3 spectrofluorimeter linked to a Zenith computer. For a fixed solution of 4 μM AT-DNA with 5 μM ethidium bromide (ethidium), the competing Pt complex **2** concentration was increased from 0 to 10 μM. The fluorescence enhancement of ethidium was measured as a function of the competing Pt complex concentration.

Ethidium shows a large fluorescence enhancement on intercalation to DNA which is directly proportional to the concentration of bound ethidium. If another ligand displaces the ethidium, then the ethidium fluorescence enhancement is reduced. If the DNA and ethidium concentrations are held constant, reduction of fluorescence enhancement may therefore be used as a direct measure of the binding of a non-fluorescent intercalating ligand. An assumption in the following analysis is that the concentration of ethidium displaced is directly proportional to the decrease in fluorescence enhancement. In fact this is not the case if energy-transfer between bound ethidium and the displacing ligand occurs, as this causes reduced fluorescence enhancement of bound ethidium [9]. This means that the bound ethidium concentration may be under-estimated and the bound Pt complex **2** concentration over-estimated, leading to an inflated value for K_p.

To extract an equilibrium binding constant, K_p from the data, the equation (see Appendix)

$$\frac{P_{tot}}{P_b^*} = \frac{K_e}{K_p}\frac{E_f}{E_b} + \frac{n_e}{n_p}$$

is used, where K_e is the equilibrium binding constant for ethidium bromide, K_p that for **2**, E_f is the concentration of free ethidium and E_b is the concentration of bound ethidium.

$$P_b^* = \frac{1}{5}\left(4E_f - E_b - 5\frac{E_b}{E_f K_e}\right)$$

for our experiment where E_{tot} = 5 μM and [DNA] = 4μM (see Appendix).

These data were collected under conditions in which the Pt complex **2** is stable, but it was shown by ^{1}H NMR spectroscopy (data not shown) that at Cl^- concentrations above about 150mM, the pyridine ligand is displaced by Cl^- to form the Pt complex **1** (R = Cl). It was also shown that mercaptoethanol rapidly (<10 min.) displaces the 4-picoline ligand from the Pt complex **2** at ambient temperature in aqueous solution.

3. Results and discussion

3.1. Synthesis of 4-picoline-2,2′:6′,2″-terpyridine-platinum(II) tetrafluoroborate **2**

A preliminary kinetic investigation of the reaction between 4-picoline and the Pt complex **1** (R = Cl) was followed by ^{1}H NMR spectroscopy, the chemical shift of the 4-methyl resonance of picoline moving downfield (about 0.3 ppm) on coordination to platinum. The reaction was complete at ambient temperature in 15–30 minutes. It has been suggested that the rapid rate of reaction in the Pt complex **1** (R = Cl) is due to the π-trans effect which labilises the Pt-Cl bond [10]. In order to isolate the Pt complex **2** it was necessary to replace the relatively nucleophilic chloride ion by a much less nucleophilic counter ion. The reaction was performed in the presence of silver tetrafluoroborate and after removal of the silver chloride, the tetrafluoroborate salt of the Pt complex **2** crystallised from solution.

The 200 MHz ^{1}H NMR spectrum of the Pt complex **2** bis-tetrafluoroborate salt in D_2O showed characteristic satellites on each side of the α protons of the 4-picoline (centred at 8.75 ppm) owing to coupling to ^{195}Pt which has a nuclear spin of 1/2 and 33.8% natural abundance. This confirmed that the Pt-picoline bond was formed, as did the electrospray mass spectrum.

3.2. Ligation assay

Unwinding – and so intercalation – was detected using an electrophoretic assay; ligation of a linear DNA molecule in the

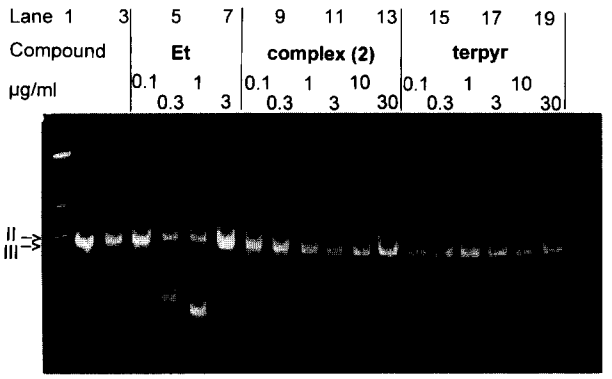

Fig. 1. The terpyr Pt (II) complex (**2**) unwinds DNA like ethidium. Linear DNA was ligated in the presence of different concentrations of ethidium (Et; lanes 4–7), terpyr Pt (II) complex (**2**) (lanes 8–13), or terpyr (lanes 14–19), the compounds removed and ligation products resolved electrophoretically; finally the gel was stained and photographed. Lane 1: /*Hin*dIII markers. Lane 2: unligated (form III) DNA. Lane 3: linear DNA ligated in the absence of any test compound. Lane 20: As lane 3, but in the presence of 1% dimethylsulphoxide, the highest concentration present during ligation. The positions of forms II and III are indicated.

presence of an unwinding agent generates a characteristic set of topoisomeric circles [5,11]. Fig. 1 illustrates a typical assay. Linear DNA runs as a single band (form III, lane 2) and most is ligated in the absence of an unwinding agent into a complicated set of products (lane 3). As some linear molecules originally contained nicks, some resulting circles are also nicked and run just behind the linear molecules; such relaxed (form II) circles constitute a background present whenever the ligase is active. However, other circles contain no nicks and run as a number of bands slightly ahead of the nicked circles. These are topoisomers containing increasing numbers of positive supercoils generated after ligation by the duplex unwinding on transfer of DNA from the ligation buffer (containing a high Mg^{2+} concentration) into the electrophoresis buffer [12]. There are additional, but minor, bands at the top of the gel formed by end-to-end ligation which give linear molecules of two or more unit lengths, together with their circular and catenated counterparts (both supercoiled and relaxed). As these forms are difficult to identify, DNA is ligated at a low concentration to minimize their formation.

If an intercalating agent such as ethidium is present during ligation, a different pattern of ligation products is obtained. At 0.1 μg/ml (Fig. 1, lane 4), there is little intercalation and the double helix is only slightly unwound. When the intercalator is removed after ligation and DNA transferred into electrophoresis buffer, the positive supercoiling due to the buffer change is offset by negative supercoiling induced by removing the intercalator. As a result, the topoisomers are less positively supercoiled than those in lane 3 and run slightly more slowly. As the concentration is increased, the negative supercoiling induced by removal of the intercalator eventually balances the positive supercoiling due to transfer between buffers; the resulting topoisomers are then centred around the mobility of the relaxed circle (not shown). At 0.3 μg/ml (lane 5), the effect of the intercalator becomes larger than the slight effects due to transfer between buffers; then, DNA unwound by intercalation, rewinds on transfer to electrophoresis buffer, so that the now negatively-supercoiled topoisomers run more rapidly as an unresolved group. After ligation in 1 μg/ml ethidium (lane 6), these topoisomers are even more negatively supercoiled; again they are not well resolved. At 3 μg/ml (lane 7), the ligase is partly inhibited, resulting in a stronger band due to (unligated) form III molecules and a weaker band of (unresolved) negatively-supercoiled topoisomers. Higher concentrations completely inhibit the ligase (not shown).

This shift – from positively supercoiled topoisomers, through a relaxed group to negatively supercoiled forms – is shown clearly by the terpyr Pt (II) complex (**2**), as its concentration is increased (lanes 8–13). Although on a weight basis the terpyr Pt (II) complex (**2**) appears to be a less powerful unwinding agent than ethidium, on a molar basis they are comparable. Again, high concentrations inhibit the ligase (lanes 11–13). Terpyridine itself does not induce these characteristic mobility changes (lanes 14–19). These results show that terpyr Pt (II) complex (**2**) – but not terpyridine – unwinds DNA like ethidium (Fig. 1). Evidence of unwinding, though circumstantial, is generally accepted as proof of intercalation [13,14]. Dithiothreitol was deliberately not added to the ligation assay as it would rapidly displace 4-picoline from the terpyr Pt (II) complex (**2**), but the enzyme preparation contains dithiothreitol and so it is likely that some, if not all, of the 4-picoline has been displaced from the terpyr Pt (II) complex (**2**) during the assay. The ligation assay cannot therefore be regarded as a true indicator of the binding strength of terpyr Pt (II) complex (**2**) to DNA.

3.3. Normal absorption studies

The electronic absorption spectrum of the Pt complex **2** has a structured charge transfer band with maxima at 350 nm, 339 nm, 324 nm that become a single unstructured smaller magnitude band with maximum at 343nm upon binding to DNA (Fig. 2).

3.4. Thermal denaturing studies

The melting curves for ct-DNA, ct-DNA with **1** (R = Cl) and ct-DNA with **2** are shown in Fig. 3 together with the derivative curves. The T_m for ct-DNA with no ligand present was 69°C, and with **1** (R = Cl) a single transition at 74°C is observed, but

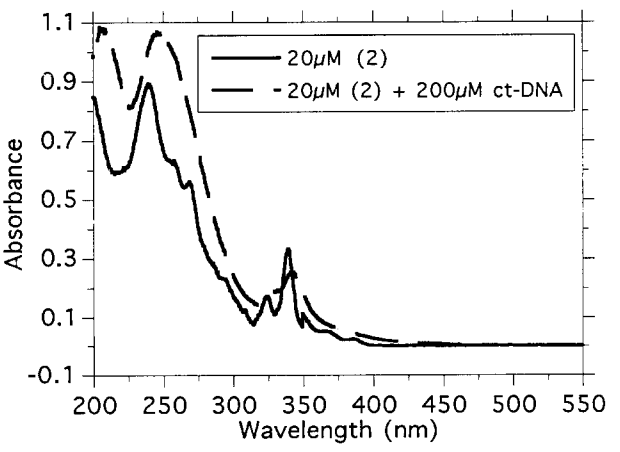

Fig. 2. Electronic absorption spectrum of 20 μM Pt complex **2**. (a) free, (b) with ct-DNA 200 μM.

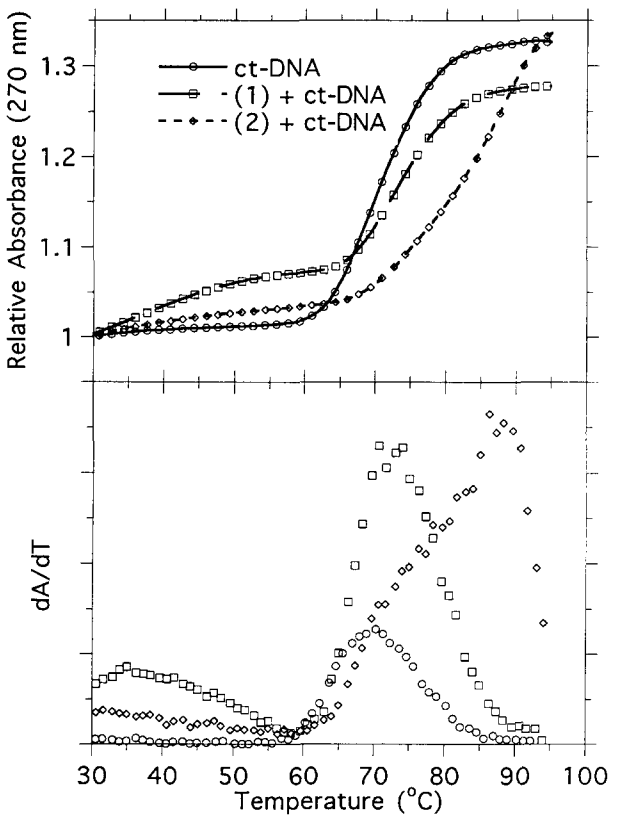

Fig. 3. Thermal denaturation curves of 400 μM ct-DNA alone (○), in the presence of 20 μM Pt complex **1** (R = Cl) (□), and 20 μM **2** (◇).

with the Pt complex **2** a first transition at approximately 75°C, was followed by a second transition at 88°C that effected complete denaturation. The double transition for the Pt complex **2** was unexpected, but as shown below (in the circular dichroism studies) the intercalation site size is 3.75 ± 0.15 base pairs in the concentration range in which the denaturing experiment was performed; this is unusually large. A possible explanation is that the ct-DNA in the region where the Pt complex **2** is not bound melts with a T_m of 75°C and the regions of the DNA into which the intercalator is bound melt at the higher transition temperature. Other explanations, however, are possible.

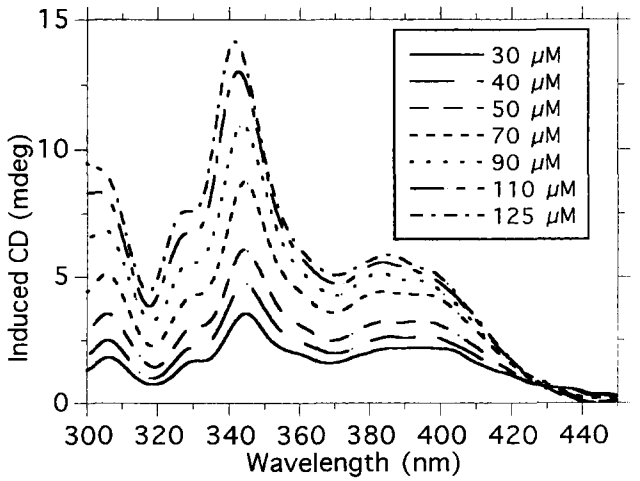

Fig. 4. ICD for 500 μM AT DNA, 0–125 μM Pt complex **2** in 10 mM NaCl.

3.5. Circular dichroism (CD) studies

As the induced spectroscpic method (ISM) relies on there being one binding mode (or constant proportions of a number of modes) present over a range of ligand concentrations, it is only expected to be applicable to regions of constant spectral form [8]. For Pt complex **2** with AT-DNA there is a change in the shape of the 500nm band when the concentration of the Pt complex reaches 90 μM (Fig. 4). The ICD value at 344 nm for concentrations less than 80 μM (Fig. 5a) and above 90 μM (Fig. 5b) were therefore used in ISM calculations. The straight line graphs in Fig. 5 confirm the uniformity of binding mode in the two concentration ranges. The results for the concentration range 30 → 80 μM are site size $n = 3.75 \pm 0.15$ base pairs and $K = (1.8 \pm 0.5) \times 10^7$ M^{-1}, and for concentration range 90 → 125μM site size $n = 2.1 \pm 0.05$ base pairs and $K = (1.17 \pm 0.09) \times 10^6$ M^{-1}. These results suggest that the doubly charged Pt complex **2**, as predicted, has a strong binding constant for

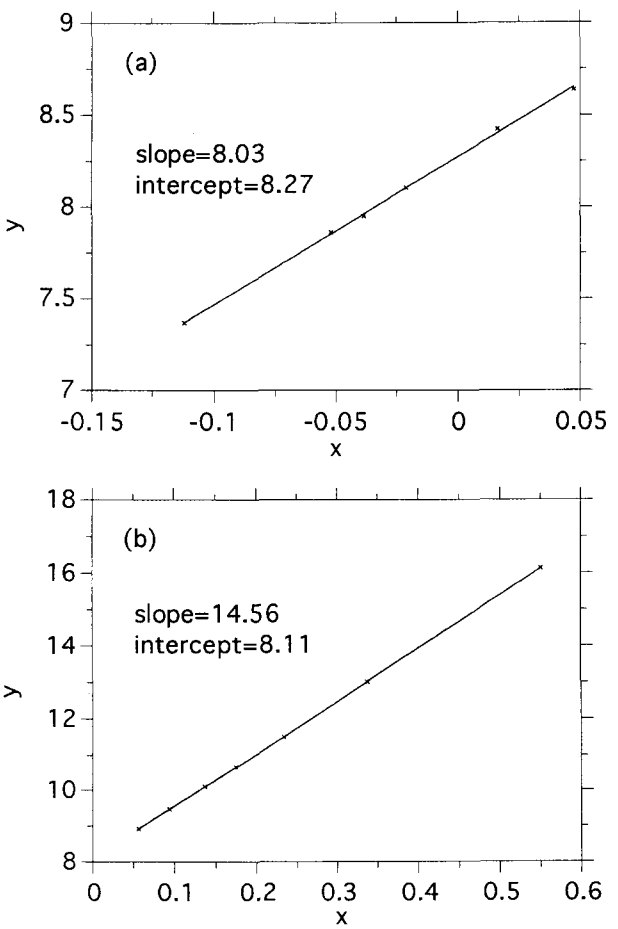

Fig. 5. Plot of $y = \delta c_i/\delta \rho_i$ versus $x = \frac{\delta(c_i/\rho_i)}{\delta \rho_i}$ for 500 μM AT-DNA with **2**. (a) 30–80 μM Pt complex **2**; (b) 90–125 μM Pt complex **2**.

AT-DNA. However, the Pt complex binds with this affinity only about every 4 base-pairs. This might be due to repulsion between the highly charged ligands. It is interesting to note that $[Ru(1,10\text{-phenanthroline})_3]^{2+}$ has the same site size at low ligand concentrations [15]. As the ratio of the Pt complex **2** to DNA is increased, a second binding mode is observed which leads to binding every 2 base pairs but with an equilibrium binding constant an order of magnitude less strong.

3.6. Fluorescence competition experiments

In order to determine the equilibrium binding constants of the Pt complex **2** to DNA independently from the CD experiments, a competition experiment (see section 2) was performed (Fig. 6). From these data $(P_t)/(P_b^*)$ vs. $(E_f)/(E_b)$ has a gradient of $(K_e)/(K_p) = (0.191 \pm 0.011)$ and an intercept $(n_e)/(n_p) = (1.09 \pm 0.14)$ (Fig. 7). Since we know K_e and n_e [9] it follows that the site size $n_p = (3.7 \pm 0.5)$ and the binding constant $K_p = (4.95 \pm 0.30) \times 10^7\ M^{-1}$ from this experiment. The value of K_p thus calculated is, as noted above, almost certainly an over-estimate of K_p owing to energy transfer from the bound ethidium to **2**. Nevertheless, it compares well with that determined by CD, confirming that the Pt complex **2** binds strongly to the DNA.

4. Conclusion

The above data demonstrate that the Pt complex **2**, like other Pt(II) terpyridines, intercalate into DNA, the first binding mode having an equilibrium binding constant of approximately $2 \times 10^7\ M^{-1}$ and a site size of about 4 base-pairs. As the ratio of the Pt complex **2** to DNA increases, a second binding mode occurs with an equilibrium binding constant of about $1 \times 10^6\ M^{-1}$ and a site size of about 2 base-pairs. It is noteworthy that the equilibrium binding constant for the first binding mode of the doubly charged Pt complex **2** is more than two orders of magnitude greater than that of the singly charged Pt complex **1** (R = SCH_2CH_2OH). This potency of binding together with the ease of synthesis makes 4-picoline-2,2′:6′,2″-terpyridine-platinum(II) **2** a valuable addition to the range of DNA binding reagents.

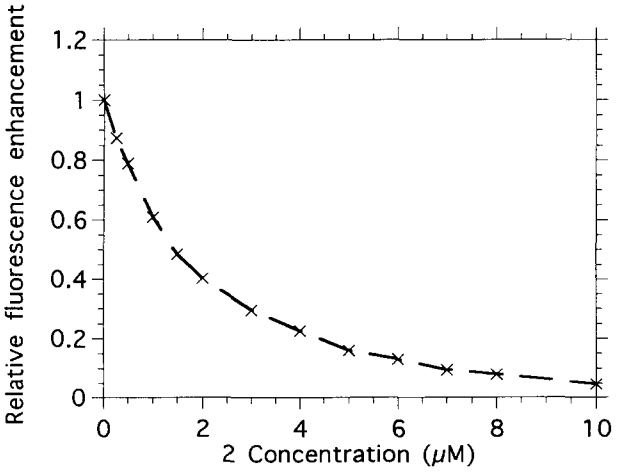

Fig. 6. Fluorescence enhancement of 5 μM ethidium and 4 μM AT-DNA with **2** in 10 mM NaCl. Excitation wavelength = 540 nm, emission wavelength = 595 nm.

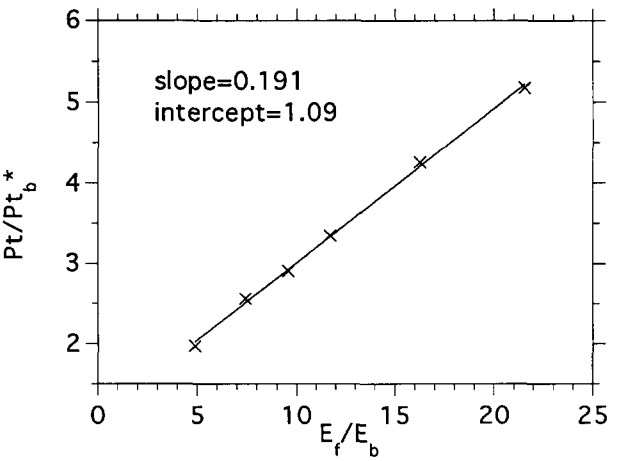

Fig. 7. P_f/P_b^* versus E_f/E_b for 5 μM ethidium and 4 μM AT-DNA with **2**.

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Appendix

The binding constant of a non-fluorescent ligand (Pt complex **2** fluorescence is extremely weak) may be determined from a competition experiment with ethidium bromide as follows. Pt complex **2** was added to a solution of ethidium bromide and DNA whose concentrations were kept constant (by adding equal volumes of Pt complex **2** solution and a DNA/ethidium bromide solution at twice the concentration of the starting solution).

An equilibrium is set up between free Pt complex **2** (P_f) plus the free DNA sites and the bound Pt complex **2** (P_b) where the concentration of free sites, S_f, is also dependent upon the ethidium bromide DNA equilibrium. So

$$K_p = \frac{P_b}{P_f S_f} \text{ and } K_e = \frac{E_b}{E_f S_f} \text{ with } S_f = \frac{E_b}{E_f K_e}\frac{n_e}{n_b}$$

(the factor n_e/n_p accounts for the difference in site sizes of the two competing intercalators). $1/K_p$ may therefore be expressed:

$$\frac{1}{K_p} = \frac{P_f E_b}{P_b E_f K_e}\frac{n_e}{n_p} = \left\{\frac{P_{tot}}{P_b} - 1\right\}\frac{E_b}{E_f K_e}\frac{n_e}{n_p}$$

so

$$\frac{P_t}{P_b} = \frac{K_e}{K_p}\frac{E_f}{E_b}\frac{n_p}{n_e} + 1$$

where E_b is assumed to be directly proportional to the fluorescence enhancement measured. $K_e = 9.5 \times 10^6\ M^{-1}$ [16]. The concentration of bound Pt complex **2**, P_b, is determined as follows.

$$n_p P_b = D - n_e E_b - n_e S_e$$

where D is the total DNA concentration, S_e are the sites free for ethidium bromide binding, $n_e E_b$ are the total sites occupied by ethidium bromide and $n_e S_e$ are the total number of free sites. For our experiment, $D = 4\ \mu M$, $E_{tot} = 5\ \mu M$ and $n_e = 4$, so

$$D = \frac{4}{5} n_e [E_b + E_f].$$

We may therefore write

$$P_b = \frac{n_e}{n_p}[P_b^*] \text{ where } P_b^* = \frac{1}{5}\left[4E_f - E_b - 5\frac{E_b}{E_f K_e}\right]$$

so

$$\frac{P_t}{P_b^*} = \frac{K_e}{K_p}\frac{E_f}{E_b} + \frac{n_e}{n_p}$$

This means that a plot of $(P_t)/(P_b^*)$ vs. $(E_f)/(E_b)$ will have a gradient of $(K_e)/(K_p)$ and an intercept of $(n_e)/(n_p)$.

Note added in proof

Linear dichroism (LD) spectra were obtained with 200 μM DNA and 0–50 μM Pt complex 2, i.e. in the lower concentration binding regime studied by CD. There is a low intensity positive LD signal at around 440 nm, the rest of the LD spectra being negative. The sample with 10 μM Pt complex 2 (where most of the ligand is bound in the first binding mode) has the highest LD value per ligand bound at all wavelengths, with a value greater than the LD value per base of pure DNA in the region of the spectrum where DNA absorbs. This indicates that the in-plane transitions of 2 are perpendicular to the DNA helix axis and that the binding of 2 stiffens or lengthens the DNA. These observations together with the unwinding assay provides powerful evidence for the intercalative mode of binding of 2 to DNA.