Interaction of phenylthiolato-(2,2',2''-terpyridine)platinum(II) cation with DNA

Laurence P. G. WAKELIN,* W. David McFADYEN,† Adam WALPOLE* and Ian A. G. ROOS*
*Experimental Chemotherapy Unit, Cancer Institute, 481 Little Lonsdale Street, Melbourne, Vic. 3000, Australia, and †Department of Chemistry and Physics, Melbourne College of Advanced Education, 757 Swanston Street, Carlton, Vic. 3052, Australia

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The interaction between a novel aromatic thiolato derivative from the family of DNA-intercalating platinum complexes, phenylthiolato-(2,2',2''-terpyridine)platinum(II)-[PhS(terpy)Pt⁺], and nucleic acids was studied by using viscosity, equilibrium-dialysis and kinetic measurements. Viscosity measurements with sonicated DNA provide direct evidence for intercalation, and show that at binding ratios below 0.2 molecules per base-pair PhS(terpy)Pt⁺ causes an increase in contour length of 0.2 nm per bound molecule. However, helix extension diminishes at greater extents of binding, indicating the existence of additional, non-intercalated, externally bound forms of the ligand. The ability of PhS(terpy)Pt⁺ to aggregate in neutral aqueous buffers at a range of ionic strengths and temperatures was assessed by using optical-absorption methods. Scatchard plots for binding to calf thymus DNA at ionic strength 0.01 (corrected for dimerization) are curvilinear, concave upward, providing further evidence for two modes of binding. The association constant decreases at higher ionic strengths, in accord with the expectations of polyelectrolyte theory, although the number of cations released per bound unipositive ligand molecule is substantially greater than 1. Stopped-flow kinetic measurements confirm the complexity of the binding reaction by revealing multiple bound forms of the ligand whose kinetic processes are both fast and closely coupled. Thermal denaturation of DNA radically alters the shapes of binding isotherms and either has little effect on, or enhances, the affinity of potential binding sites, depending on experimental conditions. Scatchard plots for binding to natural DNA species with differing nucleotide composition show that the ligand has a requirement for a single G·C base-pair at the highest-affinity intercalation sites.

The interaction of platinum(II) complexes containing 2,2'-bipyridine, 2,2',2''-terpyridine and 1,10-phenanthroline ligands with closed circular and linear DNA has been studied by Lippard and colleagues (Jennette *et al.*, 1974; Lippard *et al.*, 1976; Howe-Grant *et al.*, 1976), who have shown that each of these systems has the capacity for intercalative binding. Aliphatic thiolato derivatives of the platinum-terpyridine complex and ethylenediammine derivatives of bipyridine and phenanthroline complexes have high affinity for DNA and require a single G·C base-pair at the intercalation site (Howe-Grant *et al.*, 1976; Howe-Grant & Lippard, 1979). However, little information is available on the biological properties of metallointercalators, save the report that hydroxyethylthiolato(terpyridine)platinum inhibits genetic recombination in pneumococci (Seto & Tomaz,

1977). This paucity of biological data, together with the reported nucleotide-sequence-selectivity of platinum-containing intercalators outlined above, motivated us to synthesize diammine derivatives of platinum-bipyridine and platinum-

phenanthroline complexes as well as aromatic thiolato derivatives of the terpyridine complex. In brief, we find that platinum-phenanthroline and platinum-terpyridine complexes inhibit the growth of L1210 leukaemia cells in culture at concentrations in the micromolar range, whereas the bipyridine complexes are less active (W. D. McFadyen, L. P. G. Wakelin, I. A. G. Roos & V. A. Leopold, unpublished work). The findings suggest that the terpyridine ligands are cytotoxic, unlike the phenanthroline complexes, which appear to be cytostatic. In the present paper we describe the interaction of the parental aromatic thiolato derivative of the terpyridine complex phenylthiolato-(2,2',2''-terpyridine)platinum(II) chloride monohydrate $[\text{PhS(terpy)Pt}^+\text{Cl}^-\cdot\text{H}_2\text{O}]$ with DNA. Initially, we used optical measurements to assess the ability of the ligand to self-associate, so that Scatchard plots for binding to DNA might be corrected for dimerization of the free ligand. Equilibrium dialysis was used to investigate the relative affinities of the ligand for DNA species with differing nucleotide composition, denatured DNA and RNA. The effects of ionic strength and temperature on the binding equilibria to native and heat-denatured calf thymus DNA were studied in detail. Direct evidence for intercalation was obtained from viscosity measurements of the effect of the ligand on the relative contour length of sonicated rod-like fragments of calf thymus DNA. Finally, stopped-flow kinetic measurements were used to delineate more clearly the multifarious bound forms of the ligand revealed by the viscometric and equilibrium-binding studies.

Materials

Buffers

Experiments were conducted in Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/KOH buffers, pH 7.0 at room temperature (designated KF/Hepes buffers), containing 2 mM-Hepes (Calbiochem), 10 μM -EDTA and sufficient KF to yield the required ionic strength (9.4, 99.4 and 299.4 mM to give I 0.01, 0.10 and 0.30 respectively). Ultrapure water from a Barnstead Nanopure system was used throughout.

Nucleic acids

Escherichia coli DNA, *Micrococcus lysodeikticus* DNA, *Clostridium perfringens* DNA and calf thymus DNA were purchased from Sigma Chemical Co. Solutions containing 2 mg/ml in KF/Hepes buffer of I 0.2 were sonicated in a Branson B15 150W sonicator, fitted with the large probe, at power level 5 for a total of 2 min at 0°C. They were subsequently dialysed against buffer of the required ionic strength, clarified by filtration

through Whatman GF-C glass-fibre filters, and stored frozen at -20°C . DNA concentrations are based on an assumed $\epsilon_{(P)}_{260}$ (molar absorption coefficient with respect to nucleotide pairs) of $13200\text{M}^{-1}\cdot\text{cm}^{-1}$ for calf thymus DNA and *Cl. perfringens* DNA and on the measured value of $12600\text{M}^{-1}\cdot\text{cm}^{-1}$ for *E. coli* DNA and *M. lysodeikticus* DNA (Tubbs *et al.*, 1964). Heat-denatured calf thymus DNA was prepared by heating a solution in 0.01 I KF/Hepes buffer at 100°C for 20 min and then cooling rapidly to 0°C in an ice/water bath. High- M_r whole yeast RNA was obtained from Calbiochem. Denatured DNA and RNA solutions were dialysed exhaustively for 3 days at 4°C against the experimental buffer with the use of Spectrapor 2 dialysis tubing of M_r cut-off 12000 to ensure that the low- M_r material was removed from the samples before use in binding studies. In distinction to duplex DNA, denatured DNA and RNA concentrations are expressed with respect to nucleotides on the basis of molar absorption coefficients at 260 nm of 8200 and $8500\text{M}^{-1}\cdot\text{cm}^{-1}$ respectively (Wakelin *et al.*, 1981).

Phenylthiolato-(2,2',2''-terpyridine)platinum(II) chloride monohydrate

A 1.5-fold molar excess of thiophenol was added to a stirred suspension of $[(\text{terpy})\text{PtCl}]^+\text{Cl}^-\cdot 3\text{H}_2\text{O}$ (Howe-Grant & Lippard, 1980) in ethanol under an atmosphere of N_2 . Methanolic tetraethylammonium hydroxide, equimolar in amount to the terpyridine complex, was then added. The purple solution that resulted was filtered in air, and the product was precipitated as fine purple needles by the slow addition of diethyl ether. Analysis, calculated for $\text{C}_{21}\text{H}_{16}\text{Cl}_2\text{N}_3\text{PtS}\cdot\text{H}_2\text{O}$ (M_r 591.0): C, 42.7; H, 2.7; Cl, 6.0; N, 7.1; S, 5.4. Found: C, 42.8; H, 3.0; Cl, 6.2; N, 6.9; S, 5.4. T.l.c. showed a well-defined single spot, R_f 0.50, on fluorescent silica-gel plates in acetic acid/methanol/water (5:3:2, by vol.) as solvent. I.r.- and ^{13}C -n.m.r.-spectral measurements confirm the structure as shown. Solutions of $\text{PhS(terpy)Pt}^+\text{Cl}^-$ for binding experiments were freshly prepared or stored frozen at -20°C .

Methods

Dimerization constants

Dimerization constants for PhS(terpy)Pt^+ were determined by using the observed deviations from Beer's-Law plots of concentration versus absorbance at a wavelength of 343 nm. Absorbance measurements were made as a function of ligand concentration in 2 mm-, 10 mm- and 50 mm-light-path quartz cuvettes thermostatically controlled at 20, 37 and 52°C in a Cary 219 spectrophotometer. Assuming that PhS(terpy)Pt^+ aggregates accord-

ing to a simple dimerization model, the apparent molar absorption coefficient, $\epsilon_{app.}$, and the monomer molar absorption coefficient, ϵ_m , are related to the dimerization constant, K_d , in the following way:

$$[(\epsilon_m - \epsilon_{app.})/C_{total}]^{\frac{1}{2}} = [2K_d/(\epsilon_m - \epsilon_d)]^{\frac{1}{2}} [(\epsilon_m - \epsilon_d) - (\epsilon_m - \epsilon_{app.})]$$

where ϵ_d is the molar absorption coefficient of the dimer and C_{total} is the total drug concentration expressed in terms of the monomer M_r (Schwarz *et al.*, 1970). A plot of $[(\epsilon_m - \epsilon_{app.})/C_{total}]^{\frac{1}{2}}$ versus $(\epsilon_m - \epsilon_{app.})$ yields a straight line with intercept $[K_d(\epsilon_m - \epsilon_d)]^{\frac{1}{2}}$ on the ordinate and $(\epsilon_m - \epsilon_d)$ on the abscissa. Dimerization constants were evaluated by a linear least-squares fit to the data transformed into the form required by the above equation. Monomer molar absorption coefficients at 343 nm in buffers of 1.01, 0.10 and 0.30 were measured at concentrations in the range 2–4 μM and found to be 15500, 15300 and 14600 $\text{M}^{-1} \cdot \text{cm}^{-1}$ respectively. They were independent of temperature over the range 20–52°C.

Equilibrium dialysis

Binding curves were measured by equilibrium dialysis in an MSE Dianorm apparatus. Dialysis cells having two 5 ml compartments separated by a Spectrapor 3 regenerated cellulose membrane (nominal M_r cut-off 3500–4500) were loaded with 4 ml of nucleic acid solution in the concentration range 140–160 μM in one chamber and an equal volume of PhS(terpy)Pt⁺Cl[−] solution in the other. The cells were rotated to establish equilibrium in a water bath at 20, 37 or 52°C for 20 h, after which time the drug concentration in both chambers was determined spectrophotometrically in 50 mm- or 10 mm-light-path semimicro quartz cuvettes. To samples from each chamber was added an equal volume of dimethylformamide, and the total and free ligand concentrations in equilibrium with the DNA were established by using a molar absorption coefficient determined in buffer/dimethylformamide (1:1, v/v) mixtures at 346 nm of 16200 $\text{M}^{-1} \cdot \text{cm}^{-1}$. This molar absorption coefficient was found to be insensitive to changes in ionic strength in the range 0.01–0.30. Appropriate controls were performed to verify complete dissociation of the DNA–ligand complex by dimethylformamide over the range of binding extents studied, to establish that no nucleic acid leaked across the membrane under the conditions of the experiment and to ensure attainment of equilibrium within the 20 h period of rotation. The concentration of bound ligand, equal to the difference between total and free drug concentrations, was divided by the nucleic acid concentration to yield the binding ratio r . For experiments

with native duplex DNA the binding ratio is expressed with respect to nucleotide pairs, whereas in experiments with heat-denatured DNA and RNA it is defined with respect to nucleotides. In all cases free ligand concentrations were corrected by using the dimerization constants listed in Table 1 and values obtained for the free monomer concentration c . Binding isotherms were constructed in the form of Scatchard (1949) plots (r/c versus r).

Viscometry

Measurements were made essentially by the method of Cohen & Eisenberg (1966, 1969) in a Cannon–Ubbelohde Series 75 viscometer thermostatically maintained at $20 \pm 0.01^\circ\text{C}$ in a Cannon Instrument model M1 water bath. The viscometer was fitted with a pair of i.r. sensors that automatically trigger an electronic timing device as the meniscus of the solution passes between them, giving a flow time for water of 135 s. Flow times were measured to a precision of ± 0.02 s. The calf thymus DNA used was sonicated to fragments of M_r 2.5×10^5 – 5×10^5 (Crothers & Zimm, 1965) as previously described (Wakelin & Waring, 1976). The viscometer contained 1.0 ml of a 303 μM solution of sonicated DNA having a reduced viscosity of approx. 2 dl/g. PhS(terpy)Pt⁺Cl[−] solution was added in increments of 5–50 μl to a maximum of 250 μl from an Agla precision micrometer syringe via a fine polyethylene tube. The concentration of PhS(terpy)Pt⁺Cl[−] solution being delivered from the tip of the plastic tube was determined before and after each experiment by u.v.-absorption measurements. Solutions were freed of particulate material by passing them through 0.22 μm -pore-size Millipore filters before use. Reduced viscosities were calculated by established methods, taking into account the dilution caused by the addition of the drug solution. Values of the relative contour length of DNA in the presence of the ligand compared with free DNA, L/L_0 , were calculated as the cube root of the measured relative increase in reduced viscosity (Wakelin *et al.*, 1978).

Stopped-flow kinetics

Kinetic measurements were performed with a Dionex D110 stopped-flow spectrophotometer coupled via a d.c. amplifier and double-beam storage oscilloscope to a 64-kilobyte Apple II microcomputer equipped with two floppy-disc drives, an 8-bit analogue-to-digital converter and a high-precision clock. Software was developed to permit collection, storage, editing and analysis of up to 500 points per dataset with a wide choice of sampling frequency. The fastest rate of data collection was 0.1 ms per point. The spectrometer was

fitted with a 20mm-light-path optical cuvette, giving the apparatus a dead-time of 2ms, and was operated in transmittance mode with standard (1:1) drive syringes.

Results

Dimerization constants

The propensity of PhS(terpy)Pt⁺ to aggregate in aqueous buffers was studied under the full set of experimental conditions used to investigate binding of the ligand to nucleic acids. KF was chosen as the supporting electrolyte because of the limited solubility of the drug in buffers containing moderate to high chloride concentrations. Optical measurements were used to establish that the ligand did not hydrolyse or react with F⁻ ion under any of the experimental conditions. In all cases the values obtained for deviations from Beer's Law transformed to give linear plots (not shown) consistent with the simple dimerization model proposed by Schwarz *et al.* (1970). The measured dimerization constants are presented in Table 1, along with other thermodynamic parameters obtained from van't Hoff plots of the variation of dimerization constant with temperature. At 1.0 M K_d falls from $6.3 \times 10^3 \text{ M}^{-1}$ at 20°C to $3.5 \times 10^3 \text{ M}^{-1}$ at 52°C, revealing a small negative enthalpy change associated with a positive favourable entropy of dimerization. As the ionic strength is raised the enthalpy becomes increasingly negative, whereas there is a reversal in the sign of ΔS and entropy effects become progressively less favourable. At 20°C the dimerization constant rises with increasing ionic strength, but this phenomenon is less noticeable as the temperature is raised and it is abolished by 52°C. The dimerization constants for PhS(terpy)Pt⁺ may be compared, at low ionic strength, with the value of $7 \times 10^3 \text{ M}^{-1}$ reported for hydroxyethylthiolato(terpyridine)platinum in water at room temperature (Jennette *et al.*, 1976). Thus the

dimerization constants of the two platinum-terpyridine complexes are experimentally indistinguishable, and their tendency to self-associate is much more marked than is typically observed for phenanthridines (Wakelin & Waring, 1980) or simple acridines (Quadrifoglio *et al.*, 1974).

Interaction with calf thymus DNA

Binding isotherms for the interaction of PhS(terpy)Pt⁺ with native calf thymus DNA in buffer of 1.0 M at 20, 37, and 52°C are shown in Fig. 1. In all three cases the isotherms are markedly curvilinear (concave upwards), indicative of strong anti-cooperativity and/or heterogeneity of binding sites having differing affinities (Crothers, 1968; Gurskii *et al.*, 1972; McGhee & von Hippel, 1974). Thus the data are not amenable to simple Scatchard analysis, nor could the results be fitted by eqn. (10) of McGhee & von Hippel (1974), which corresponds to an excluded-site model. In this situation the intrinsic association constant to the highest-affinity potential binding site, $K_{(0)}$, can be estimated by extrapolating to the ordinate axis (Crothers, 1968; Muller & Crothers, 1968; McGhee & von Hippel, 1974), yielding values for $K_{(0)}$ of 3.5×10^5 , 3.0×10^5 and $3.0 \times 10^5 \text{ M}^{-1}$ at 20, 37 and 52°C respectively (Table 2). Hence at low ionic strength the binding constant is independent of temperature and the interaction is driven entirely by favourable entropy effects. The isotherms in Fig. 1 also show that binding ratios in the range 0.5–1.0 ligand molecules per base-pair are readily attained at values of r/c well above zero, which, taking into consideration the neighbour-exclusion principle (Crothers, 1968), implies that a second non-intercalative mode of binding along the surface of the helix is available to PhS(terpy)Pt⁺. This suggestion finds further support from measurements of the effect of the ligand on the contour length of sonicated rod-like fragments of calf thymus DNA. Fig. 2 shows that for binding

Table 1. Thermodynamics of dimerization of PhS(terpy)Pt⁺

Dimerization constants were evaluated from deviations from Beer's Law as described in the Methods section by using the equation of Schwarz *et al.* (1970). Enthalpies of dimerization were obtained from van't Hoff plots of the variation of K_d with temperature, and entropy effects were calculated by using the equations $\Delta G = -RT \cdot \ln K_d$ and $\Delta G = \Delta H - T\Delta S$. The uncertainty in values of K_d is estimated to be $\pm 10\%$.

Ionic strength	Temperature (°C)	K_d (M ⁻¹)	ΔG (kJ/mol)	ΔH (kJ/mol)	ΔS (J·mol ⁻¹ ·K ⁻¹)
0.01	20	6.3×10^3	-21	-14	25
0.01	37	5.3×10^3	-22	-14	27
0.01	52	3.5×10^3	-22	-14	25
0.10	20	1.1×10^4	-23	-34	-40
0.10	37	5.8×10^3	-22	-34	-39
0.10	52	2.7×10^3	-21	-34	-40
0.30	20	1.8×10^4	-24	-38	-47
0.30	37	7.3×10^3	-23	-38	-48
0.30	52	3.9×10^3	-22	-38	-47

Fig. 1. Interaction between PhS(terpy)Pt^+ and calf thymus DNA in buffer of $I\ 0.01$

(a) Measurements made at 20°C ; (b) measurements made at 37°C ; (c) measurements made at 52°C . The data are plotted in accordance with Scatchard (1949), where r is the binding ratio (ligand molecules bound per nucleotide pair) and c is the concentration of free monomeric ligand.

Fig. 2. Effect of PhS(terpy)Pt^+ on the relative contour length of sonicated rod-like fragments of calf thymus DNA in buffer of $I\ 0.01$ at 20°C

The ordinate represents the calculated ratio of contour lengths in the presence (L) or in the absence (L_0) of the ligand. The abscissa represents the binding ratio expressed as PhS(terpy)Pt^+ molecules bound per base-pair. The continuous line of slope 0.59 is a linear least-squares fit to the initial portion of the curve. A slope of 1 is equivalent to a helix extension of 0.34nm ($3.4\text{\AA}$) per bound ligand molecule.

ratios up to 0.2 there is a linear increase in helix extension of 0.2nm ($2.0\text{\AA}$) per bound ligand molecule, which compares with the value of 0.27nm ($2.7\text{\AA}$) reported for the intercalation of ethidium and proflavine (Reinert, 1973). However, at

greater extents of binding the increase in contour length diminishes, and the value of L/L_0 reaches a plateau by about 0.5 ligand molecules bound per base-pair. This suggests that at the highest binding ratios PhS(terpy)Pt^+ binds in a non-intercalative fashion that is not associated with a lengthening of the helix.

Increasing the ionic strength severely weakens the affinity of PhS(terpy)Pt^+ for DNA as is shown in Figs. 3 and 4, where the binding data are fitted to eqn. (10) of McGhee & von Hippel (1974). At $I\ 0.1$ and $0.3\ K_0$ decreases at elevated temperatures. The enthalpy of binding becomes increasingly negative and the value of ΔS falls as the salt concentration rises, and thus the position of equilibrium is less dominated by entropy effects under these experimental conditions (see Table 2). Bearing in mind the complex nature of the modes of binding of the ligand, and the fact that the data extend over only a limited portion of the binding curve, the values for the number of nucleotides per binding site given in Table 2 should be interpreted with caution.

It is instructive to analyse the ionic-strength-dependence of the affinity constants according to the polyelectrolyte theory developed by Manning (1969) and Record *et al.* (1978). Fig. 5 shows the predicted linear relationship between $\ln K_0$ and $\ln I$, with the slopes of the straight lines indicating the release of 1.4, 1.4 and 1.6K^+ ions per bound ligand molecule at 20, 37 and 52°C respectively. Extrapolating the data to 1M-KF , with appropriate corrections for activity coefficients at this salt con-

Fig. 3. Scatchard plots for binding of PhS(terpy)Pt^+ to calf thymus DNA in buffer of $I\ 0.10$
For details see the legend to Fig. 1. The continuous curves are theoretical, computed to fit eqn. (10) of McGhee & von Hippel (1974) with the use of the parameters for $K_{(0)}$ and N in Table 2.

Fig. 4. Interaction between PhS(terpy)Pt^+ and calf thymus DNA in buffer of $I\ 0.30$
For details see the legend to Fig. 3.

Table 2. *Parameters of binding for the interaction of PhS(terpy)Pt⁺ with calf thymus DNA*

With the exception of measurements at *I* 0.01, where association constants were determined by linear extrapolation of binding data to the Scatchard-plot ordinate, binding parameters were evaluated by using a non-linear least-squares fit to eqn. (10) of McGhee & von Hippel (1974):

$$r/c = K_{(0)}(1 - Nr) \left[\frac{(1 - Nr)}{(1 - (N-1)r)} \right]^{N-1}$$

Enthalpies and entropies of reaction were determined as described in the legend to Table 1. The uncertainty in values of $K_{(0)}$ is estimated to be $\pm 10\%$.

Ionic strength	Temperature (°C)	$K_{(0)}(\text{M}^{-1})$	<i>N</i> (nucleotide pairs per binding site)	ΔG (kJ/mol)	ΔH (kJ/mol)	ΔS ($\text{J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$)
0.01	20	3.5×10^5	—	−31	−3.9	92
0.01	37	3.0×10^5	—	−32	−3.9	92
0.01	52	3.0×10^5	—	−34	−3.9	92
0.10	20	1.8×10^4	1.3	−24	−10	47
0.10	37	1.4×10^4	1.8	−25	−10	47
0.10	52	1.2×10^4	2.4	−25	−10	47
0.30	20	5.9×10^3	1.0	−21	−16	16
0.30	37	4.3×10^3	1.4	−22	−16	17
0.30	52	3.0×10^3	2.1	−22	−16	16

Fig. 5. *Ionic-strength-dependence of the affinity of PhS(terpy)Pt⁺ for calf thymus DNA*

Symbols: ○, measurements made at 20°C; □, measurements made at 37°C; △, measurements made at 52°C. The ■ symbol represents the values of the binding constant at 37 and 52°C, and the continuous lines are linear least-squares fits to the experimental points.

ethidium and other phenanthridines and acridines (Wakelin & Waring, 1980), and reveals that the extent of reaction is now overwhelmingly determined by a negative enthalpy of 19 kJ/mol associated with small unfavourable entropy effects (ΔS approx. $-2 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$). Comparison with the similarly corrected measurements made by Howe-Grant & Lippard (1979) for binding of hydroxyethylthiolato(terpyridine)platinum to DNA ($K_{(0)}^{1M} = 2.3 \times 10^3 \text{ M}^{-1}$) shows that replacing the aliphatic thiol with thiophenol has little, if any, effect on the intrinsic stability of the platinum–terpyridine intercalation complex.

The kinetics of binding of PhS(terpy)Pt⁺ to calf thymus DNA were investigated by using absorbance measurements at 343 nm. At ionic strengths in the range 0.01–1.0 at 20°C the association reaction was found to be too fast to give time-resolvable optical effects under pseudo-first-order conditions (final DNA concentration 100 μM , final ligand concentration 10 μM) in the stopped-flow spectrophotometer. Cooling the reaction to 9°C at *I* 0.05 slows the association rate sufficiently to allow observation of a single exponential transient of time constant 2.2 ms. Raising the concentration of the DNA to 200 μM and that of the ligand to 20 μM has no effect on this time constant, revealing that the transient represents a first-order process with a rate constant of $4.6 \times 10^2 \text{ s}^{-1}$. At both DNA concentrations the amplitude of the measured exponential accounts for only 5–10% of the total absorbance change on binding, confirming that bimolecular association is still too fast to measure

centration, facilitates calculation of the affinity of PhS(terpy)Pt⁺ for DNA in the absence of ion condensation effects, $K_{(0)}^{1M}$. Table 3 summarizes the findings that at *I* 1.0 the binding constants are an order of magnitude less than those measured for

Table 3. Interpretation of the ionic-strength-dependence of the parameters of binding of PhS(terpy)Pt^+ to calf thymus DNA according to polyelectrolyte theory

The slopes of the plots in Fig. 5 have been divided by the factor 0.88 to yield the number of counter-ions released per bound ligand molecule (Record *et al.*, 1978). Extrapolation of the data to $\ln I = 0$, after appropriate corrections for activity coefficients in 1M-KF, gave the binding constants in the absence of ion condensation effects $K_{(0)}^{1M}$. Enthalpies and entropies of reaction at this ionic strength were determined as described in the legend to Table 1. The uncertainty in values of $K_{(0)}^{1M}$ is estimated to be $\pm 10\%$.

Temperature (°C)	Slope of $\ln K_{(0)}$ - versus- $\ln I$ plot	Counter-ions released	$K_{(0)}^{1M}$ (M^{-1})	ΔG^{1M} (kJ/mol)	ΔH^{1M} (kJ/mol)	ΔS^{1M} ($J \cdot mol^{-1} \cdot K^{-1}$)
20	1.21	1.4	1.7×10^3	-19	-19	-2.3
37	1.26	1.4	1.2×10^3	-18	-19	-1.7
52	1.36	1.6	8.1×10^2	-18	-19	-2.3

and indicating that the observed process characterizes a monomolecular re-arrangement of bound PhS(terpy)Pt^+ molecules. Dissociation rates were measured directly by using the detergent-sequestration technique (Fox *et al.*, 1981), with the anionic surfactants Sarkosyl (sodium salt of *N*-lauroylsarcosine) and Gardisperse A.C. (sodium nonylphenoxy-pentaethoxysulphate; Albright and Wilson). The use of sodium dodecyl sulphate was precluded because of its limited solubility in buffers containing greater than 0.01 M-KF. DNA-ligand complexes comprising 200 μM -DNA and 10 μM - PhS(terpy)Pt^+ in buffer of ionic I 0.05 were mixed with equal volumes of 20mM-detergent in the same buffer: thus the final concentrations were 100 μM -DNA, 5 μM -ligand and 10mM-detergent. The two detergents gave experimentally indistinguishable results, and only the mean values of the time constants are presented. At 20°C the observable dissociation reaction is described by a single exponential of time constant 3.5ms. However, it is clear from the amplitude of the transient, compared with the absorbance change characterizing complete dissociation, that the major part of the reaction has taken place at a faster rate within the 2ms dead-time of the instrument. This complexity of reaction was confirmed at 9°C, where the time-resolvable absorbance change is 3-fold larger and is composed of two closely coupled exponential effects of equal amplitude. Thus at the lower temperature the reaction has slowed sufficiently to bring most of the reaction into the stopped-flow time scale, revealing dissociation steps with time constants of 1.8ms and 8.6ms. That the binding mechanism is even yet more complex, however, is apparent from the fact that the total absorbance change of the observed processes still accounts for only half the amount of the ligand bound. Given that there are multifarious bound forms of PhS(terpy)Pt^+ , it is difficult to evaluate the observed transients directly in terms of rate constants. This problem is exacerbated by the finding that the kinetic steps are closely

Fig. 6. Scatchard plots for binding of PhS(terpy)Pt^+ to heat-denatured calf thymus DNA in buffer of I 0.10. The binding ratio is defined as the number of ligand molecules bound per nucleotide. Symbols: ●, measurements made at 20°C; ○, measurements made at 37°C; Δ, measurements made at 52°C. The continuous curves are theoretical, computed to fit eqn. (10) of McGhee & von Hippel (1974) with the use of the parameters for $K_{(0)}$ and N listed in Table 4.

coupled, and thus the transients most probably represent combinations of rate constants.

Interaction with heat-denatured DNA

To investigate the influence of polynucleotide secondary structure on the interaction of PhS(terpy)Pt^+ with DNA, binding curves were measured with heat-denatured calf thymus DNA. Fig. 6 shows Scatchard plots determined in buffer of I 0.1 at 20, 37 and 52°C, in which the binding ratio is

expressed with respect to nucleotides because of uncertainty in the nature of the binding site. At 20°C the affinity for DNA is only marginally affected by denaturation, although the number of binding sites is drastically decreased, suggesting that the ligand interacts with residual duplex regions (Table 4). However, on raising the temperature to 37°C the binding constant increases significantly and the number of binding sites almost doubles, indicating the appearance of a new structural feature with high affinity for the ligand. This structure appears to be reasonably labile, since by 52°C both the affinity and the number of sites is halved compared with the values obtained at 37°C, and the binding constant falls to a lower value than obtained at 20°C (Table 4).

In an effort to shed further light on whether the ligand interacts with single-stranded or residual duplex regions of denatured DNA, binding experiments were conducted in the presence of formaldehyde, which is known to disrupt secondary structure (McGhee & von Hippel, 1977). Fig. 7 shows binding curves to denatured DNA at I 0.1 in the presence of 0.32M-formaldehyde, the denatured DNA having previously been incubated with 0.64M-formaldehyde for either 3 h at 25°C (○ symbols) or for 1 h at 50°C (△ symbols). Neither treatment has abolished binding, the former leaving the affinity unchanged compared with measurements made in the absence of formalde-

Fig. 7. Interaction of PhS(terpy)Pt^+ with heat-denatured calf thymus DNA in buffer of I 0.1 containing 0.32M-formaldehyde

Symbols: denatured DNA pretreated with 0.64M-formaldehyde for 3 h at 25°C, (○) or for 1 h at 50°C (△). Binding measurements were made at 20°C. For other details see the legend to Fig. 6.

hyde (Table 4), and the latter decreasing the binding constant 2-fold. It appears that the number of sites has risen significantly in both cases (Table 4), but the limited range of the data must cast some doubt on the reliability of the estimates of the binding-site size. Thus it is clear from these measurements that, in addition to duplex DNA, single-stranded DNA also provides a good template for binding PhS(terpy)Pt^+ .

The ionic-strength-dependence of $K_{(0)}$ for denatured DNA further reveals the existence of special structures with high affinity for the ligand in disorganized polynucleotides (see Fig. 8 and Table 4). By contrast with native DNA, the binding constant at 20°C for interaction with denatured DNA does not vary monotonically with salt concentration, having the values of 5.9×10^5 , 1.6×10^4 and $6.3 \times 10^4 \text{ M}^{-1}$ at I 0.01, 0.1 and 0.3 respectively. The tighter binding at I 0.3 than at I 0.1 is reminiscent of the appearance of the high-affinity site observed at 37°C in 0.1 M salt, and suggests that a similar structural feature may also occur at lower temperatures in buffer of I 0.3. All attempts to

Fig. 8. Scatchard plots for binding of PhS(terpy)Pt^+ to heat-denatured calf thymus DNA in buffers of I 0.01 and 0.3

Symbols: △, measurements made at I 0.01, $m=5$; ○, measurements made at I 0.3, $m=4$. The temperature was 20°C. For other details see the legend to Fig. 6.

Table 4. *Parameters of binding of interaction of PhS(terpy)Pt⁺ with heat-denatured calf thymus DNA*
 Binding parameters were evaluated by using a non-linear least-squares fit to eqn. (10) of McGhee & von Hippel (1974) as described in the legend to Table 2. Values of $K_{(0)}$ and N are expressed with respect to nucleotides, and details of the formaldehyde treatment are given in the text. The uncertainty in values of $K_{(0)}$ is estimated to be $\pm 10\%$.

Ionic strength	Temperature (°C)	Formaldehyde treatment	$K_{(0)}$ (M^{-1})	N (nucleotides per binding site)
0.01	20	None	5.9×10^5	3.2
0.10	20	None	1.6×10^4	5.2
0.10	37	None	2.3×10^4	2.5
0.10	52	None	1.1×10^4	5.9
0.10	20	0.64 M for 1 h at 50°C	8.4×10^3	1.8
0.10	20	0.64 M for 3 h at 25°C	1.5×10^4	3.3
0.30	20	None	6.3×10^4	6.1

Fig. 9. Scatchard plots for binding of PhS(terpy)Pt⁺ to bacterial DNA species in buffer of I 0.1 at 20°C (a) *M. lysodeikticus* DNA; (b) *E. coli* DNA; (c) *Cl. perfringens* DNA. The continuous lines are theoretical, computed to fit eqn. (10) of McGhee & von Hippel (1974) with the use of the parameters for $K_{(0)}$ and N listed in Table 5.

measure binding curves to RNA were frustrated by precipitation of the complex at ionic strengths in the range 0.01–0.3. However, limited data were obtained at low binding ratios in buffer of I 0.1 at 20°C, which indicate that the association constant is approx. $1 \times 10^4 M^{-1}$.

Interaction with bacterial DNA species of differing nucleotide composition

The capacity of PhS(terpy)Pt⁺ to discriminate between different nucleotide sequences was investigated by measuring binding curves to

bacterial DNA species of different nucleotide compositions. Fig. 9 shows Scatchard plots for interaction with DNA from *M. lysodeikticus* (72% G+C), *E. coli* (50% G+C) and *Cl. perfringens* (30% G+C) in buffer of I 0.1 at 20°C. It is evident that the binding constant diminishes as the G+C content of the DNA is decreased. The degree of selectivity for G·C base-pairs may be made more explicit by evaluating the parameter α , defined as the ratio of affinity constant for DNA X to the affinity constant for DNA Y (Muller & Crothers, 1975), and comparing it with the corresponding

Table 5. Parameters of binding of PhS(terpy)Pt^+ to DNA species of different nucleotide composition

Binding parameters were evaluated by using a non-linear least-squares fit to eqn. (10) of McGhee & von Hippel (1974) as described in the legend to Table 2. The values of $K_{(0)}$ and N are expressed with respect to nucleotide pairs. All measurements were made in buffer of I 0.1 at 20°C. $\alpha_{\text{obs.}}$ is given by the ratio of $K_{(0)}$ for the DNA in question to $K_{(0)}$ for *Cl. perfringens* DNA, and $\alpha_{\text{calc.}}$ is given by the ratio of the G + C content of the DNA in question to that of *Cl. perfringens* DNA. The uncertainty in values of $K_{(0)}$ is estimated to be $\pm 10\%$.

DNA	G + C content (%)	$K_{(0)}$ (M^{-1})	N (nucleotide pairs per binding site)	$\alpha_{\text{obs.}}$	$\alpha_{\text{calc.}}$
<i>Cl. perfringens</i>	30	1.4×10^4	1.3	—	—
Calf thymus	42	1.8×10^4	1.3	1.4	1.4
<i>E. coli</i>	50	2.2×10^4	1.9	1.7	1.7
<i>M. lysodeikticus</i>	72	3.4×10^4	2.1	2.6	2.4

Fig. 10. Dependence of the binding constant of PhS(terpy)Pt^+ on nucleotide composition of DNA

Data are taken from Table 5 and represent binding measurements made in buffer of I 0.1 at 20°C.

ratio of probability of occurrence of a G·C base-pair in an intercalation site for DNA species X and Y . Table 5 records the parameters of binding of the ligand to bacterial and calf thymus DNA species together with values of α calculated with respect to the binding constant for *Cl. perfringens*. The theoretical value expected for α , assuming that only one G·C base-pair is required in the intercalation site, is given by the ratio of the G + C content of the DNA in question to that of *Cl. perfringens* DNA. There is good agreement between the observed values of α and the values calculated in this manner (Table 5), implying that the highest-affinity sites contain at least one G·C base-pair. That a single G·C base-pair is both a necessary and sufficient condition for binding is confirmed by the linear dependence of affinity on the G + C content of the DNA, the regression line of which passes through the origin (Fig. 10).

Discussion

Interaction with calf thymus DNA

The equilibrium isotherms obtained at I 0.01, taken together with the result of the helix-extension measurements, provide firm evidence that PhS(terpy)Pt^+ intercalates into DNA at low binding ratios and that external binding predominates at high degrees of binding. Thus the broad modes of interaction of the ligand with DNA are similar to those found for the classical intercalating agent proflavine (Blake & Peacocke, 1968). The strength of intercalative binding is substantial, the binding constant of $3.5 \times 10^5 \text{ M}^{-1}$ at I 0.01 being equivalent to that of 9-aminoacridine under the same conditions (Wilson *et al.*, 1981). Although the intercalative binding constant is virtually independent of temperature at I 0.01, as is the case for ethidium (Le Pecq & Paoletti, 1967), the shapes of the Scatchard plots are, by contrast, quite sensitive to variations in temperature. There is clear suppression of the isotherms at high binding ratios as the temperature is raised from 20°C through 37°C to 52°C (Fig. 1): thus external attachment of the ligand to the DNA is accompanied by a negative enthalpy of binding. The curvature in the helix-extension plot (Fig. 2) is difficult to account for with certainty, since its origins may be ascribed to several different effects. For example, external binding may become significant at binding ratios above 0.2; the intercalated ligand may induce kinking of the helix above this degree of binding (see Wakelin *et al.*, 1981); or there may be a change in intrinsic extension per bound ligand molecule as the lattice becomes saturated.

The mechanism of binding to DNA is shown by the kinetic measurements to be both fast and complex. The bimolecular association rate constant is greater than $10^7 \text{ M}^{-1} \text{ s}^{-1}$ at temperatures above 9°C, and once bound the ligand undergoes rapid ($\tau = 2.2 \text{ ms}$) monomolecular interconversion even at low degrees of binding ($r = 0.05$). Thus the kinetic mechanism is also similar to that found for

the interaction of proflavine with DNA (Li & Crothers, 1969). Dissociation measurements confirm the kinetic complexity and reveal that there are at least three distinguishable PhS(terpy)Pt^+ -DNA complexes on the pathway from bound to free ligand at $r = 0.05$. However, to what extent these complexes ($\tau_0 < 1 \text{ ms}$, $\tau_1 = 1.8 \text{ ms}$ and $\tau_2 = 8.6 \text{ ms}$ at 9°C) are associated with parallel or sequential steps in the reaction is unclear, as is their relative occupancy at equilibrium. More-detailed studies are required to answer these questions, but, because of the rapid equilibria involved, such investigations would necessitate the use of faster kinetic methods, e.g. temperature-jump relaxation spectroscopy.

Evaluation of the ionic-strength-dependence of the binding parameters in the framework of polyelectrolyte theory (Manning, 1969; Record *et al.*, 1978) reveals a peculiarity also shared by the aliphatic thiolato derivative hydroxyethylthiolato-(terpyridine)platinum (Howe-Grant & Lippard, 1979), namely that although the ligands are unipositive ions each releases more than one condensed cation from DNA on binding (see Table 3). For the hydroxyethanethiol derivative the excess cation release (1.5 cations per bound ligand) was attributed to hydrogen-bonding interactions of the hydroxy group with DNA phosphate groups (Howe-Grant & Lippard, 1979). It is clear, however, that this explanation cannot be extended to the example of the thiophenol derivative. In the absence of ligand-anion ion-pair formation (see below), the phenomenon implies that the charge density of the helix in the vicinity of the complex is lowered more than is predicted by theory, or found for other intercalating agents (Record *et al.*, 1978; Wilson & Lopp, 1979; Wilson *et al.*, 1981). Since the helix-extension measurements do not speak for abnormal structural perturbations to the DNA duplex, it is possible that the decrease in charge density is achieved by orbital mixing and charge transfer between the base-pairs and the electron-deficient platinum-terpyridine system. If so, the dependence of cation release on temperature would imply a greater extent of electron delocalization at higher temperatures. It seems unlikely that the apparent excess cation release is attributable to the concurrent release of anions bound to the free metalointercalator on complexing with DNA (Record *et al.*, 1978). Binding data for the aliphatic and aromatic thiolato compounds have been obtained in buffers containing NaCl (Howe-Grant & Lippard, 1979) and KF (the present work) respectively, yet both intercalators exhibit comparable amounts of excess cation release. If anion binding were the root cause of the phenomenon, then this would suggest that Cl^- and F^- ions bind with similar affinity to platinum-terpyridine com-

plexes. That this is not the case, at least in the solid state, is demonstrated by the very limited solubility of PhS(terpy)Pt^+ in NaCl solutions of concentration 0.1 M or higher, which contrasts sharply with its solubility in buffers containing KF, NaF and NH_4F .

Interaction with denatured calf thymus DNA

Although many of the binding properties for the interaction of PhS(terpy)Pt^+ with native DNA are reminiscent of those that characterize the interaction of proflavine and 9-aminoacridine with duplex DNA, binding of the metalointercalator to denatured DNA differs from that of the simple aminoacridines. On denaturation the association constant falls and the number of sites rises for proflavine and 9-aminoacridine (Wilson *et al.*, 1981), whereas for PhS(terpy)Pt^+ the situation is more complex, but on the whole the number of sites diminishes and the binding constant remains unchanged or is found to increase (cf. Tables 2 and 4). Indeed, the decrease in the number of sites and the enhancement of affinity is quite marked at I 0.01 and 0.3, being particularly so at the higher salt concentration. Both the peculiar dependence of $K_{(0)}$ on experimental conditions, where, for example, the affinity is greater at 37°C than at 20 and 52°C at I 0.1, and at 20°C it is larger at I 0.3 than at I 0.1, and the dependence of the number of sites on the temperature in buffer of I 0.1, where the number at 37°C is more than double that at 20 and 52°C , strongly imply that there are special structural features recognized by the ligand in single-stranded DNA polymers. The results of measurements performed in the presence of formaldehyde strongly implicate truly single-stranded regions of denatured DNA as sites of high-affinity binding, although the curious variations of binding parameters with ionic strength and temperature do not always support this conclusion.

Interaction with DNA species of differing base composition

Association constants for interaction with DNA species of different nucleotide compositions clearly show that PhS(terpy)Pt^+ intercalates with highest affinity into a site that contains at least one G·C base-pair. The same selectivity is shared by the hydroxyethanethiol analogue (Howe-Grant & Lippard, 1979), implying that the requirement for a G·C base-pair in the binding site is an intrinsic property of the intercalating platinum-terpyridine moiety. This degree of selectivity places these ligands in the middle of the spectrum for intercalating agents, being more selective, for example, than ethidium, proflavine and ellipticine (Muller & Crothers, 1975), and less G·C-base-pair-specific than echinomycin (Wakelin & Waring, 1976) and

methyated derivatives of Phenyl Neutral Red (Muller *et al.*, 1975). Ligands with the greatest nucleotide-specificity, echinomycin and Phenyl Neutral Red among others, encompass two or three G·C base-pairs at their binding sites (Muller *et al.*, 1975; Wakelin & Waring, 1976). Muller & Crothers (1975) have proposed that G·C-base-pair-specificity arises from coupled steric and electronic factors, which combine to maximize monopole-monopole interactions between the ligand and the base-pair. They observe that specificity is found among highly methylated intercalating agents, and suggest that monopole interactions are greatest when the ligand is oriented so that bulky substituents (e.g. the dimethylamino groups of Acridine Orange) lie in the major groove of DNA. They extend this notion to the case of highly G·C-base-pair-selective Phenyl Neutral Red derivatives and similarly position the phenyl ring in the major groove (Muller *et al.*, 1975). It may be noted that in the crystal structure of the hydroxyethylthiolato(terpyridine)platinum-deoxy-CpG intercalation complex (Wang *et al.*, 1978) the thiolato tail is located in the wide groove of the mini DNA helix and the platinum atom lies sandwiched directly between the O-6 oxygen atoms of the guanine residues. This is the precise orientation that maximizes the monopole-monopole interaction between the positively charged platinum atom (formal charge +2) and the large residual negative charge on the O-6 atom of guanine (Dean & Wakelin, 1979). Experiments with Corey-Pauling-Koltun molecular models indicate that PhS(terpy)Pt⁺ also binds with less steric hindrance when the thiophenol residue lies in the wide groove, once again providing a complex with the necessary geometry for optimizing monopolar forces. In addition to these purely electrostatic interactions, it should be borne in mind that specificity may also have a quantum chemical component associated with charge transfer from the electron-rich G·C base-pair to the cationic platinum atom mediated via π -orbital mixing with the terpyridine system.

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