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A thermodynamic and spectroscopic study on the binding of berenil to poly d(AT) and to poly (dA) · poly (dT)

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Abstract

The complete thermodynamic profile for the non-intercalative binding of berenil to the alternating copolymer poly d(AT) and to the homopolymer poly (dA) · poly (dT) was investigated. Differential Scanning Calorimetry (DSC) and UV absorbance spectroscopy have been used to characterize and to compare the binding of berenil to the different synthetic polymers. Both double stranded DNA's show two types of binding; one stronger binding mode at low berenil concentrations and a weaker, in the case of poly d(AT)–berenil complexes slightly cooperative binding mode at higher drug to base pair ratios. For the interaction of berenil with poly d(AT) the thermodynamic data $\Delta G^0_{\text{bind}} = -33$ kJ/mol drug, $\Delta H^0_{\text{bind}} = -29$ kJ/mol of drug and $\Delta S^0_{\text{bind}} = +13$ J/Kmol of drug were calculated. For the minor groove binding of berenil to poly (dA) · poly (dT) the following values were obtained: $\Delta G^0_{\text{bind}} = -34$ kJ/mol of drug, $\Delta H^0_{\text{bind}} = -25$ kJ/mol of drug and $\Delta S^0_{\text{bind}} = +30$ J/Kmol of drug. Temperature-dependent UV absorbance spectroscopy revealed for both duplexes a biphasic "melting" behavior. However, the saturated nucleic acids (drug to base pair ratio 0.33) "melted" monophasically and with a decreased length of the cooperative unit. The obtained apparent equilibrium constants K_{app} for the complexation with the bischarged drug molecule showed to be a sensitive function of the ionic environment. But in contradiction to the expected release of two counterions into the solvent only a value of 1.0 was observed for the alternating copolymer poly d(AT). The complexation of berenil with poly (dA) · poly (dT) is followed by a release of 1.4 ions indicating stronger electrostatic interactions. For both polynucleotides the predicted release of two ions is not achieved. This is due to the presence of a binding mode, which involves less electrostatic interactions. From the complete data set it is proposed that the mode of binding is closely related to that found for the analogue minor groove binders DAPI and netropsin.

Keywords: Berenil; Poly d(AT); Poly (dA) · poly (dT); Differential scanning calorimetry; UV absorbance spectroscopy; Scatchard plot

1. Introduction

Specific interactions between nucleic acids and antibiotics play a central role in many pharmacologically important reactions. Berenil (1,3-bis(4′-amidinophenyl)triazene) (Fig. 1) represents a drug belonging to the group of aromatic diamidines which exert mild cytotoxic, antibacterial and antiviral properties [2,3,5,9,26,37] without having any significant antileucemic activity [2,3]. The compound shows a marked preference for A,T-rich DNA sequences and is supposed to interact by at least two hydrogen bonds in addition to electrostatic interactions with the two terminal amidine groups [2,5,7,11,15,26,27,36,37]. For DNA's in the classical B-conformation the reported width of

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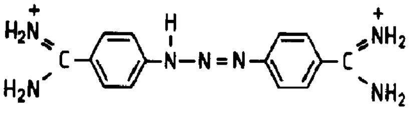

Fig. 1. Structure of the diarylamidine berenil (1,3-bis(4′-amidinophenyl)triazene).

the minor groove adopts values between 9.2 Å and 12 Å [11]. Thus the drug molecule berenil is able to penetrate deeply with its concave side into the groove. It was suggested that this deep penetration may influence the high selectivity for A,T-rich DNA domains because in G,C-regions the exocyclic 2-amino-group of guanine prohibits this kind of interaction. It is important to note that the triazene NH-group is located on the convex side of the molecule and hence is not involved in the hydrogen bonding [12]. Several authors reported a slightly altered secondary structure of the nucleic acids upon drug binding without a concomitant change in the tertiary structure [4,14,34].

It is well known that the synthetic polymer poly d(AT) usually exists in the typical B-conformation, whereas the homopolymer poly d(A) · poly d(T) adopts a heterogeneous conformation with one strand in the conformation of the A-genus and the other strand in a B-conformation [1,26,33]. Thus the conformation of the DNA's may also reflect an interesting feature in the understanding of the selectivity of minor groove binders [15]. The detailed knowledge of the thermodynamic profile and the binding parameters should give a closer insight into the nature of drug binding. For this reason a thorough investigation of the site-sizes and the association constants for the binding of berenil to synthetic polymers under varying experimental conditions seemed to be important.

2. Materials and methods

Poly d(AT) and poly (dA) · poly (dT) were purchased from Sigma. The polynucleotides were dissolved in a solution containing 100 m*M* NaCl, dialysed against water and lyophilized. One day prior to utilization the samples were redissolved in the appropriate buffer (2 m*M* sodium phosphate, 0.1 m*M* EDTA, 10-250 m*M* NaCl, pH 7.0) and incubated at 37°C for 1 h. Unless otherwise stated the buffer containing 10 m*M* NaCl was used. Berenil was obtained from Sigma and used without further purification. The concentration of the polynucleotides was determined at 25°C spectrophotometrically using the following extinction coefficients: poly d(AT), $\varepsilon_{260} = 6650$ cm^{-1} M^{-1} phosphate; poly (dA) · poly (dT) $\varepsilon_{260} = 6500$ cm^{-1} M^{-1} phosphate. The berenil concentrations were obtained by carefully weighing the dried powder or spectrophotometrically by using an extinction coefficient of $\varepsilon_{368} = 31000$ cm^{-1} M^{-1}.

2.1. UV absorbance spectroscopy

Spectrophotometric measurements were performed with a Perkin-Elmer Lambda 7 UV-Vis Spectrometer. Temperature was controlled with a home-built digital controller. The temperature-induced UV "melting" profiles were registered at 260 nm with a x-y recorder and digitized as ASCII-files. The heating rate in all experiments was 1°C/min. In all experiments a berenil solution of an equal concentration as in the polynucleotide-drug solution was used as the reference. The contribution of the absorbance difference between bound and free berenil at 260 nm is negligible compared to the DNA absorbance and therefore the temperature-induced dissociation of the ligand did not significantly disturb the transition curves. This was proved by the same hyperchromicity values of the free and complexed polynucleotides due to the "melting" of the base pairs. The light-path of the used cuvettes was 0.1, 0.5 or 1 cm.

2.2. Spectral titrations

Binding isotherms of the berenil binding to poly d(AT) and poly (dA) · poly (dT) were obtained spectrophotometrically [35]. Upon binding the absorbance maximum of berenil shifts from 368 nm to 381 nm, accompanied by a decrease of the extinction coefficient from 31000 cm^{-1} M^{-1} to 24300 cm^{-1} M^{-1}. The concentration of bound berenil (D_b) was obtained by measuring the absorbance difference $\Delta \mathrm{Abs}_{402}$ between the polynucleotide–berenil complex and a berenil solution of equal concentration at 402 nm. The concentration of bound berenil is then given by

$$D_b = \Delta \mathrm{Abs}_{402} / [\varepsilon_{b,402} - \varepsilon_{f,402}]d, \qquad (1)$$

where d equals the light path of the cuvettes. The extinction coefficients at 402 nm of bound and free berenil $\varepsilon_{b,402}$ and $\varepsilon_{f,402}$ were determined as described elsewhere [34] and were $\varepsilon_{b,402} = 18900 \pm 2\%$ and $\varepsilon_{f,402} = 9700 \pm 2\%$ at 298 K.

The free berenil concentration D_f was calculated by subtracting the amount of bound ligand D_b from the total berenil concentration D_t. The titrations were performed by mixing equal volumes of polynucleotide solution (1.42×10^{-4} M) with various berenil solutions of appropriate concentrations (1.38×10^{-6} M–7.1×10^{-5} M). After mixing and prior to the measurements the solutions were incubated 30 min at 310 K.

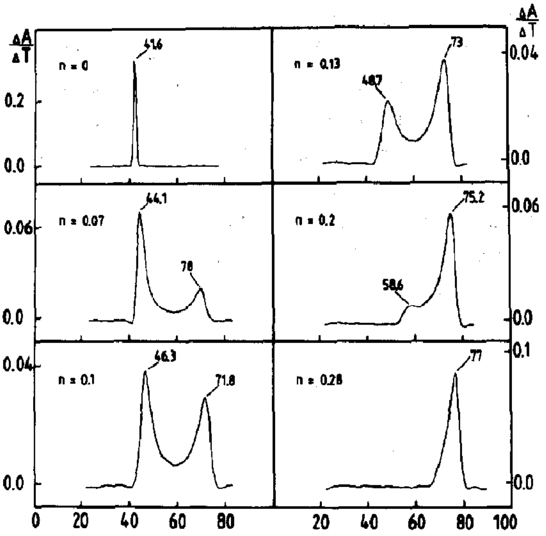

Fig. 2. Differentiated UV transition curves of poly d(AT)–berenil complexes for various drug to base pair ratios n measured at 260 nm. The polynucleotide concentration was 7.12×10^{-5} M. The "melting" temperatures of the helix-to-coil transitions are indicated.

2.3. Differential scanning calorimetry (DSC)

The DSC-experiments were performed on a DASM-4 microcalorimeter (V/O Mashpriborintorg, Russia) equipped with a serial input–output system for data collection developed in our laboratory [28]. The temperature was increased by a rate of 1°C /min.

3. Results

3.1. Temperature-dependent UV absorbance

A characterization of the thermal stability of nucleic acids is easily achieved by monitoring the temperature-induced helix-to-coil transition. The binding of berenil results in a stabilization of the helix, which consequently "melts" at a higher temperature T_m. In the case of incomplete saturation this results in a biphasic temperature–absorbance profile. From the differentiated UV absorbance versus temperature plots the transition temperatures can easily be derived [19–21]. Saturation is indicated by a monophasic transition at high temperature with a slope of the differentiated curve nearly similar to that obtained for the uncomplexed DNA [25]. For both polymers saturation was accomplished at a drug to base pair ratio of 0.33. The differentiated transition curves for berenil binding to poly d(AT) at various drug to base pair ratios n in Fig. 2 clearly demonstrate the stabilization of the free and the complexed base pairs by increasing the drug concentration. The measurement of ΔT_m (T_m of stabilized duplex saturated with berenil minus T_m^0 of drug free duplex) allows the calculation of the overall apparent binding constant K_{app} according to the theory applied by Crothers [8]. The shift in T_m as a consequence of ligand binding can be described by eq. (2):

$$\frac{1}{T_m^0} - \frac{1}{T_m} = \frac{1}{N_1} \frac{R}{\Delta H^{\mathrm{DSC}}} \ln[1 + K_{\mathrm{app}} D_f] \qquad (2)$$

where T_m^0 and T_m are the Kelvin temperatures corresponding to the midpoints of the helix to coil transitions in the absence and in the presence of ligand, respectively. The enthalpy difference ΔH^{DSC} for the melting of the ligand-free base pair was obtained by differential scanning calorimetry [21]. R is the gas constant, $1/N_1$ is the ratio of bound ligand molecule per base pair at saturation and is taken from the Scatchard plot according to the procedure described in Section 3.2. D_f represents the free ligand concentration in the measured solution at the transition temperature T_m. To achieve saturation a 50% excess of ligand concentration was used in all experiments. K_{app} is the apparent equilibrium constant for the association of berenil with the macromolecule. For comparison the K_{app}-values, corresponding to the high "melting" temperatures (T_m), were extrapolated to 298 K following the standard thermodynamic relationship:

$$\frac{\partial \ln K}{\partial T} = \frac{\Delta H_{bind}}{RT^2} \tag{3}$$

Here ΔH_{bind} is the enthalpy of the association, which can be calculated as described in Section 3.4. In Tables 1 and 2 for both polymers and their berenil complexes the apparent equilibrium constants calculated using eq. (2) and the "melting" temperatures (T_m, T_m^0) are given for various salt concentrations. From the temperature versus UV absorbance plots it is possible to evaluate the $\Delta H_{v.H.}^{UV}$-values according to the all-or-none model described by Marky and Breslauer [22]. Transforming the "melting" profile to an Θ versus T plot (Θ = degree of helix formation) allows calculating $\Delta H_{v.H.}^{UV}$ from the slope at T_m by application of the eqs. (4) and (5):

$$\Delta H_{v.H.}^{UV} = 6RT_m^2\left(\frac{\partial \Theta}{\partial T}\right)_{T=T_m} \tag{4}$$

for the free duplex and

$$\Delta H_{v.H.}^{UV} = 8RT_m^2\left(\frac{\partial \Theta}{\partial T}\right)_{T=T_m} \tag{5}$$

for the ternary complex. The results obtained by eq. (4) are also listed in Tables 1 and 2, respectively.

Table 1

UV determined association constants K_{app} and transition temperatures T_m of saturated poly d(AT)–berenil complexes at various sodium concentrations. Also listed are the transition temperatures T_m^0 and the van 't Hoff enthalpies $\Delta H_{v.H.}^{UV}$ of the free poly d(AT) duplex at the indicated sodium concentrations

[Na$^+$] [M]	K_{app} (298 K) (M^{-1})	T_m (°C)	T_m^0 (°C)	$\Delta H_{v.H.}^{UV}$ free duplex (kJ/mol coop. unit)
0.016	2.4×10^6	78.0	41.6	−2605
0.036	1.3×10^6	78.5	49.4	−2485
0.066	6.5×10^5	77.1	54.3	−2283
0.086	4.3×10^5	76.2	57.0	−1855
0.116	3.6×10^5	77.5	59.7	−1827
0.156	2.8×10^5	75.3	62.9	−1725
0.206	2.5×10^5	76.7	63.9	−1547
0.256	2.0×10^5	77.0	65.6	−1509

3.2. Scatchard procedure

In order to obtain binding constants describing the interaction of small ligands with polymers several methods have been developed [24,30–32]. If the amount of bound ligand can be determined experimentally, usually plots according to the procedure described by Scatchard [31] are used to extract the binding data. The desired value r was calculated according to $r = D_b/c_{AT}$ (c_{AT} = concentration of polynucleotide in base pairs) and plotted against r/D_f. The values D_f and D_b were determined as outlined in Section 2.2. The resultant Scatchard plots for the berenil–poly d(AT) and berenil–poly (dA)·poly (dT) interaction showed a concave curvature (Fig. 3). Non-linear Scatchard plots may indicate more than

Table 2

UV determined association constants K_{app} and transition temperatures T_m of saturated poly (dA)·poly (dT)–berenil complexes at various sodium concentrations. Also listed are the transition temperatures T_m^0 and the van 't Hoff enthalpies $\Delta H_{v.H.}^{UV}$ of the free poly (dA)·poly (dT) duplex at the indicated sodium concentrations

[Na$^+$] (M)	K_{app} (298 K) (M^{-1})	T_m (°C)	T_m^0 (°C)	$\Delta H_{v.H.}^{UV}$ free duplex (kJ/mol coop. unit)
0.016	3.8×10^6	86.3	48.8	−6290
0.036	1.5×10^6	86.1	56.5	−6210
0.086	4.1×10^5	84.1	65.0	−6185
0.116	3.1×10^5	84.4	67.5	−6070
0.156	2.7×10^5	84.8	69.6	−5902
0.256	1.4×10^5	84.7	74.1	−5728

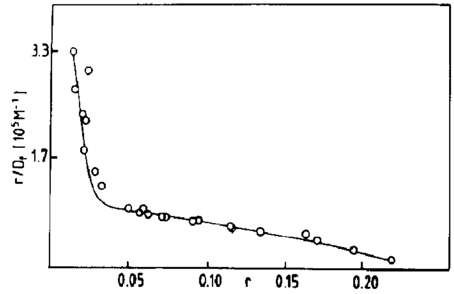

Fig. 3. Scatchard plot of berenil binding to poly d(AT) at 10 mM NaCl, 2 mM sodium phosphate and 0.1 mM EDTA at pH 7. Each point is an average obtained by two independent measurements. The polymer concentration was 7.12×10^{-5} M (in base pairs). D_f is the concentration of unbound berenil and r the moles of bound berenil per base pair. The curve is constructed according to the parameters obtained by a simulation fitting best the experimental data points.

one class of binding modes, interaction between the ligands or may be the result of ligands covering more than one repeating unit on the one-dimensional polymer lattice (overlapping site) as it is expected for berenil [24,32]. Certainly also a combination of this features is possible. Considering only one binding mode the theoretical approach of McGhee and von Hippel [24] takes the phenomena of overlapping binding sites and cooperatively into account. Thus we initially used this method to evaluate the binding parameters. The overall binding equation, eq. (6), is:

$$\frac{r}{D_f} = K(1-Nr) \cdot \left(\frac{(2\alpha+1)(1-Nr)+r-R}{2(\alpha-1)(1-Nr)} \right)^{N-1} \cdot \left(\frac{1-(N+1)r+R}{2(1-Nr)} \right)^{2} \qquad (6)$$

with R defined by eq. (7):

$$R = \sqrt{\left([1-(N+1)r]^2 + 4\alpha r(1-Nr) \right)}. \qquad (7)$$

In the case of non-interacting ligands eq. (8) is obtained:

$$\frac{r}{D_f} = K(1-Nr) \cdot \left(\frac{1-Nr}{1-(N-1)r} \right)^{N-1}, \qquad (8)$$

where N denotes the size of the site given in the number of base pairs covered by one ligand; K is the intrinsic association constant of the site and α denotes the cooperativity exponent. An exponent of $\alpha = 1$ implicates no ligand interaction and eq. (6) reduces to eq. (8). For $0 < \alpha < 1$ the binding is anticooperative and α-values > 1 represent positive cooperativity.

To extract the desired binding parameters N, K and α the following analysis was used. Based on the experimental data points a theoretical curve was calculated. The calculation was accomplished using a modified spline-function [38] which resulted in a smoothed curve minimizing the scattering of the experimental data points. A further advantage of this procedure resulted in an increased number of data points and consequently in better fits by the Marquardt–Levenberg method [38]. The choice of the starting parameters was very important to yield good fits due to the tight coupling of the binding parameters K and α [13,24]. Estimates of the parameters were obtained using the following procedure:

The most simple consideration was a single, non-interacting binding mode. Following the theory of McGhee and von Hippel [24] a least-squares fit to the curve at low r-values to a tangent line with an y-intercept which gave an estimated K-value for $r \rightarrow 0$. The r-intercept of this tangent line is defined as $1/(2N-1)$. This value allows an estimate of N. The r-intercept of the binding isotherm itself is given by $1/N$ which is in practice difficult to estimate because the predicted plots are essentially flat for $N > 5$ [24]. Attempts to fit the experimental data assuming one non-interacting binding mode (eq. 8) and using the initial parameters N and K were not successful. Fitting of the binding isotherm according to eq. (6) with different values of α between 0 and 1 (anticooperativity) failed as well. Thus we concluded that at least two different binding modes are present.

The approach of McGhee and von Hippel is not suitable for systems involving more than one binding mode. Especially we have to consider mutual exclusion which means that only one ligand per site is possible at a time. An appropriate theory for more than one binding mode including the concept of mutual exclusion was used to determine the desired binding parameters [32]. For m different binding modes the calculation of a binding isotherm is accomplished using eq. (9):

$$\frac{r}{D_f} = \sum_{i=1}^{m} \frac{K_i}{s_i N_i} U_i \qquad (9)$$

with

$$U_i = \frac{\Phi_i}{1 + \sum_i^m \Phi_i} \quad \text{and} \quad s_i = K_i D_f.$$

In eq. (9) K_i is equivalent to $\alpha_i K_i^*$ where K_i^* is the nucleation binding constant for an isolated bound ligand on the polymer lattice. The Φ_i-function is defined as the ratio of occupied vs. unoccupied binding contacts and can be calculated according to eq. (10):

$$\Phi_i = \frac{N_i \alpha_i s_i \lambda^{N_i + 1}}{\left[\alpha_i \lambda^{N_i} - (\alpha_i - 1) s_i\right]^2}. \qquad (10)$$

In this equation λ is calculated by means of an iterative procedure as described by Schwarz [32]. Rough estimates of the characteristic binding parameters were obtained from the practical Scatchard plot as outlined elsewhere [32] and refined by curve fitting [38].

The described procedure was applied to the experimental data of both synthetic polymers at 10 mM and 150 mM NaCl-concentration. In all cases the complexation showed two distinct binding modes with intrinsic association constants K_2 of $\sim 10^6$ to $\sim 10^5$ M^{-1} and K_1-values of $\sim 10^5$ to $\sim 10^4$ M^{-1}. The determined values of site-sizes in base pairs were $N_2 > 8$ and $N_1 \sim 3$, respectively. Figure 3 shows the Scatchard plot of berenil binding to poly d(AT) at 10 mM NaCl. Table 3 summarizes the results for berenil binding to poly d(AT) and to poly (dA)·poly (dT) at the indicated sodium concentrations. The site-sizes are given as integer-values of the number of covered base pairs by one drug molecule.

Table 3

Binding parameters for the berenil–polynucleotide complexes at the indicated NaCl concentrations. The parameters α_1, N_1, N_2, K_1 and K_2 were obtained by the Scatchard-procedure described in the text

	poly d(AT)–berenil complex	poly (dA)·poly (dT)–berenil complexes
10 mM NaCl		
α_1	3.4	1
N_1	3	3
N_2	23	70
K_1	4.5×10^5 M^{-1}	8.7×10^4 M^{-1}
K_2	5.2×10^5 M^{-1}	9.3×10^5 M^{-1}
150 mM NaCl		
α_1	1.4	40 (α_2)
N_1	4	3
N_2	35	8
K_1	1.9×10^5 M^{-1}	1.4×10^4 M^{-1}
K_2	8.2×10^4 M^{-1}	4.7×10^5 M^{-1}

3.3. *Salt-dependence of the association constant*

At neutral pH berenil possesses two positively charged amidino-groups. If these terminal groups electrostatically contribute to the minor groove binding, counterions of the polymeric phosphate backbone should be released to the solvent. This effect would result in a significant salt-dependence of the association constant. Following the theoretical treatment of Record [29] and Manning [17] a log–log plot of K_{app} versus the molar cation concentration should give a straight line. The plot is described by eq. (11):

$$\log K_{app} = \log K^0 - (Z\Psi + k) \log \mathrm{M}^+. \qquad (11)$$

Z is the valence of the ligand interacting with Z phosphates of the nucleic acid. M^+ represents the cation concentration and Ψ is a function of the axial charge density of the polymer. For double-stranded DNA, $\Psi = 0.88$ [29]. The number of anions released are represented by k. The constant K^0 represents the equilibrium:

$$\mathrm{Ber} + \mathrm{DNA} \overset{K^0}{\rightleftharpoons} [\mathrm{Ber{-}DNA}] + (Z\Psi + k)\mathrm{M}^+.$$

Table 4 shows the data obtained at 298 K.

To elucidate if there is any anion release from the ligand upon binding an additional experiment was performed. K_{app} was measured as a function of [(n-butyl)$_4$NCl] at constant sodium concentra-

Table 4

Slope and y-intercept data, obtained according to eq. (11), describing the salt-dependence of the berenil-polynucleotide complexes

	poly d(AT)–berenil complex	poly (dA)·poly (dT)–berenil complex
$(Z\Psi + k)$	−0.92	−1.22
$\log K^0$	+4.73	+4.39
$(Z\Psi + k)$[a]	−0.49	−0.76

[a] Values derived from experiments at constant NaCl-concentration (150 mM) and different [(n-butyl)$_4$NCl]-concentrations.

tion. The use of [(n-butyl)$_4$NCl] allows the variation of the anion concentration without affecting the extend of the condensed ions on the polymeric backbone. The experiment was carried out with a constant NaCl-concentration of 150 mM and [(n-butyl)$_4$NCl]-concentrations ranging from 10 mM to 120 mM. Higher tetrabutylammoniumchloride concentrations lead to substantial errors due to the high ionic strength. Figure 4 shows the results of these experiments for both polymers.

3.4. Differential scanning calorimetry (DSC)

The calorimetric measurements of helix-to-coil transitions for the free and ligand-saturated polynucleotides allows calculating the binding enthalpy (ΔH^0_{bind}). From a straightforward analysis

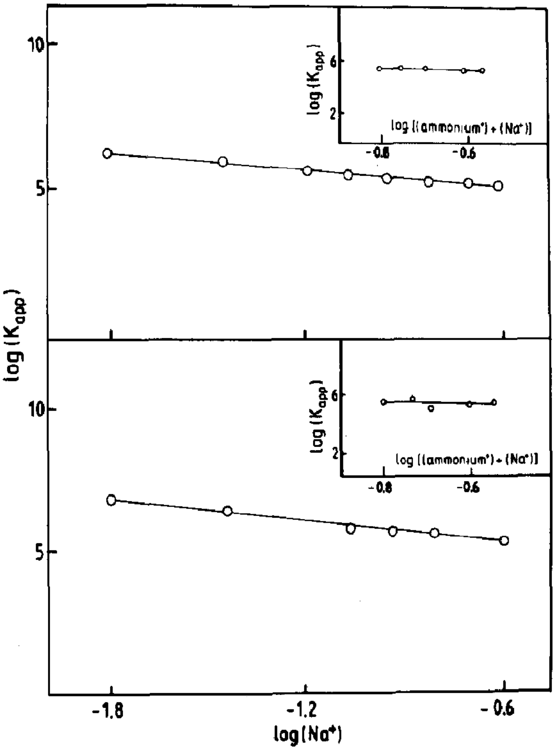

Fig. 4. Top: The salt-dependence of the association constant for the binding of berenil to poly d(AT) at 298 K is shown in a $\log K_{\text{app}}$ vs. $\log[\text{Na}^+]$ plot according to eq. (11). The poly d(AT) concentration was 4.13×10^{-5} M and the drug to base pair ratio 0.5. The inlet shows the salt-dependence using a constant NaCl concentration of 150 mM and various (n-butyl)$_4$NCl concentrations. Bottom: Results obtained for poly (dA)·poly (dT)–berenil complexes. The polymer concentration was 3.8×10^{-5} M in all experiments.

Table 5

Thermodynamic data for the drug free and complexed polymers at 10 mM NaCl

Quantity	Poly d(AT)	Poly d(AT)–berenil complex	Poly (dA)·poly (dT)	Poly (dA)·poly (dT)–berenil complex
T_m(DSC)	42.9°C	83.1°C	48.7°C	89.7°C
T_m(UV)	42.0°C	83.1°C	48.6°C	88.6°C
ΔH^{DSC}	−30.3 (kJ/mol)	−39.9 (kJ/mol)	−34.3 (kJ/mol)	−42.7 (kJ/mol)
$\Delta H^{\text{DSC}}_{\text{v.H.}}$	−2790 (kJ/mol)	−818 (kJ/mol)	−6240 (kJ/mol)	−984 (kJ/mol)
$\Delta H^{\text{UV}}_{\text{v.H.}}$	−2605 (kJ/mol)	n.d.[a]	−6290 (kJ/mol)	n.d.[a]
σ^{DSC}	92	21	182	23
ΔH^0_{bind}	−28.8 (kJ/mol drug)		−25.2 (kJ/mol drug)	
$\Delta G^0_{\text{bind,scat}}$	−32.6 (kJ/mol drug)		−34.1 (kJ/mol drug)	
$\Delta G^0_{\text{bind,UV}}$	−36.4 (kJ/mol drug)		−37.6 (kJ/mol drug)	
$\Delta S^0_{\text{bind,scat}}$	+12.7 (J/K mol drug)		+29.8 (J/K mol drug)	
$\Delta S^0_{\text{bind,UV}}$	+25.5 (J/K mol drug)		+41.6 (J/K mol drug)	

[a] n.d.: not determined.

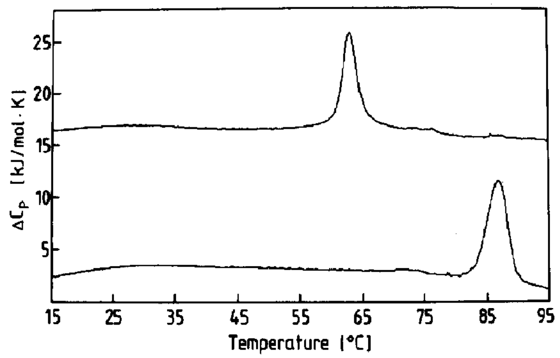

Fig. 5. Top: Temperature-induced helix-to-coil transition curve of poly d(AT) in 2 mM sodium phosphate, 150 mM NaCl and 0.1 mM EDTA at pH 7. The calorimetric molar heat capacity is shown vs. the temperature. The polymer concentration was 7.45×10^{-4} M. Bottom: Transition of the berenil saturated duplex at a drug to base pair ratio of 0.5 and experimental conditions as mentioned above.

the transition enthalpy for the free duplex (ΔH_f^{DSC}) and for the saturated duplex (ΔH_t^{DSC}) can be derived [22]. The binding enthalpy is obtained by subtracting ΔH_f^{DSC} from ΔH_t^{DSC} according to Breslauer et al. [9]. A drug to base pair ratio of 0.5 was adjusted in all experiments. The van 't Hoff transition enthalpy $\Delta H_{v.H.}^{DSC}$ for the complexation reaction can be calculated according to the eqs. (4) and (5) similar to the procedure outlined in Section 3.1. In this case $(\partial\Theta/\partial T)_{T=T_m}$ is directly taken from the slope of an Θ versus T plot, which can readily be derived from the calorimetric transition curves. Θ represents the degree of helix formation.

The ratio $\sigma^{DSC} = \Delta H_{v.H.}^{DSC}/\Delta H^{DSC}$ provides a measure of the size of the cooperative unit [20,22]. Figure 5 shows the calorimetric transition curve of free poly d(AT) and the transition of the poly d(AT)–berenil complex at saturation. Table 5 gives an overview of the obtained data for the drug-free and the stabilized polynucleotides at 16 mM Na$^+$. The standard free enthalpy ΔG_{bind}^0 can be calculated from the equilibrium constants according to $\Delta G^0 = -RT \ln K$. Under the conditions used throughout the experiments even at high drug to base pair ratios the stronger binding modes contribute with their higher association constants K_i to the overall binding. According to this a rough comparison of the K_{app}-values determined following eq. (2) (Tables 1 and 2) with the higher K_i-values obtained by the Scatchard-procedure (Table 3) might be allowed. The ΔG^0-values derived from these K_i-values and from the K_{app}-values determined by eq. (2) are listed ($\Delta G_{bind,scat}^0$, $\Delta G_{bind,UV}^0$) in Table 5 for 10 mM NaCl. Using the standard thermodynamic relationship $\Delta G^0 = \Delta H^0 - T\Delta S^0$ the binding entropies ($\Delta S_{bind,scat}^0$, $\Delta S_{bind,UV}^0$) were calculated.

Table 6

Thermodynamic data for the drug free and complexed polymers at 150 mM NaCl

Quantity	Poly d(AT)	poly d(AT)–berenil complex	poly (dA)· poly (dT)	poly (dA)· poly (dT)–berenil complex
T_m(DSC)	63.2°C	87.0°C	70.7°C	92.4°C
T_m(UV)	62.9°C	75.3°C	69.6°C	84.8°C
ΔH^{DSC}	−29.6 (kJ/mol)	−39.2 (kJ/mol)	−34.8 (kJ/mol)	−45.4 (kJ/mol)
$\Delta H_{v.H.}^{DSC}$	−1701 (kJ/mol)	−2066 (kJ/mol)	−6700 (kJ/mol)	−1717 (kJ/mol)
$\Delta H_{v.H.}^{UV}$	−1725 (kJ/mol)	n.d. [a]	−5902 (kJ/mol)	n.d. [a]
σ^{DSC}	57	53	192	38
ΔH_{bind}^0	−28.8 (kJ/mol drug)		−31.8 (kJ/mol drug)	
$\Delta G_{bind,scat}^0$	−30.1 (kJ/mol drug)		−32.3 (kJ/mol drug)	
$\Delta G_{bind,UV}^0$	−31.1 (kJ/mol drug)		−31.0 (kJ/mol drug)	
$\Delta S_{bind,scat}^0$	0.0 (J/K mol drug)		0.0 (J/K mol drug)	
$\Delta S_{bind,UV}^0$	+7.7 (J/K mol drug)		−2.7 (J/K mol drug)	

[a] n.d.: not determined.

Table 6 shows the complete data set obtained at 150 m*M* NaCl.

4. Discussion

The binding of berenil to the alternating copolymer and to the homopolymer at 16 m*M* and 156 m*M* sodium concentration clearly shows two distinct binding modes. This is comparable to the analogues DAPI [18] and hydroxystilbamidine [10]. At 10 m*M* NaCl one binding mode is slightly cooperative (poly d(AT)–berenil complex) and characterized by a site occupying ~ 3 base pairs. This binding mode appears for higher drug to base pair ratios with an equilibrium constant K_1 of ~ 10^5 M^{-1}. At low drug to base pair ratios berenil binds to both polynucleotides preferentially with a site-size of more than 20 base pairs and a stronger K_2 of ~ 10^6 M^{-1} (Table 3).

Generally, if there exists more than one binding mode, it can be shown, that for $r \to \infty$ in the original Scatchard plot the *x*-intercept is determined by the smallest *N*-value [32]. In other words saturation at very high ligand concentrations is achieved by the binding mode with the minimum site-size. Consequently under these conditions (which can be unattainable in practice) a prediction of the selectivity of berenil binding to the investigated polymers is possible by comparing the association constants K_1 (Table 3). For berenil binding to the alternating polymer the determined value of 4.5×10^5 M^{-1} is five times larger than the value of 8.7×10^4 M^{-1} derived for berenil binding to poly (dA) · poly (dT) exhibiting a higher selectivity of berenil for the alternating polymer poly d(AT). An analogue observation was made using footprinting experiments [27]. However, at low ligand concentrations ($r \to 0$) calculating the individual binding curves of mode 1 and 2 (data not shown) demonstrate that the main contribution to the binding reaction is given by mode 2. Thus, at small ligand concentrations the K_2-values of 5.2×10^5 M^{-1} for berenil binding to the alternating copolymer and 9.3×10^5 M^{-1} for poly (dA) · poly (dT)–berenil complexes indicate a greater selectivity for the homopolymer. Obviously, if there is more than one binding mode it is of major importance to know the detailed binding parameters for discussing selectivities. Depending on the drug to base pair ratio a binding mode can be substantially suppressed, regardless of the value of the association constant which then could lead to confusional results in the discussion of selectivities.

To elucidate the salt-dependence of the binding process Scatchard plots from experiments in a buffer containing 150 m*M* NaCl were investigated (Table 3). Under these conditions and for high ligand concentrations ($r \to \infty$) the obtained K_1-value of berenil binding to the alternating polymer reveals a higher selectivity compared to the homopolymer poly (dA) · poly (dT). The association constant of 1.9×10^5 M^{-1} in complexes of berenil and poly d(AT) is approximately a factor of 14 larger than the K_1-value of 1.4×10^4 M^{-1} obtained for the binding of berenil to the homopolymer (Table 3). At low berenil concentrations ($r \to 0$) the K_i^*-values have to be compared. Under these conditions cooperative ligand interaction becomes negligible and the association constant should be equal to the nucleation binding constant K_i^* calculated as K_i/α_i. Thus for the binding of berenil to poly (dA) · poly (dT) a value of $K_i^* \sim 10^4$ M^{-1} is compared to ~ 10^5 M^{-1} in berenil–poly d(AT) complexes showing a preference for the alternating polymer.

In poly d(AT)–berenil complexes increasing the salt concentration from 10 m*M* to 150 m*M* decreases the cooperativity exponent α_1 slightly from 3.4 to 1.4. The value K_2 which contributes substantially to the overall binding behavior at 10 m*M* NaCl and small berenil concentrations is lowered by a factor of 6 from 5.2×10^5 M^{-1} to 8.2×10^4 M^{-1}, thus exhibiting a low but significant salt-dependence. Hence this binding mode must involve electrostatic interactions, while for the weaker binding mode electrostatic contributions are of minor importance (K_1 varies by a factor of ~ 2).

In the case of berenil binding to poly (dA) · poly (dT) the increased salt concentration results in a different binding behavior. The Scatchard plot shows a bell-shaped curvature indicating a highly cooperative interaction of the ligand (data not shown). For the binding mode 2 a cooperativity exponent α_2 of 40 and a K_2^* of 1.2×10^4 M^{-1} was obtained. Thus in contrast to the behavior of berenil binding to the alternating polymer the association constant K_2^* depends seriously (factor of ~ 77) on the salt concentration. Upon increasing the salt concentration the binding parameter K_1 of mode 1 which should determine the binding behavior at high ligand concentrations ($r \to \infty$) changes only slightly (factor of ~ 6 in the binding constant). Similar results were reported for the analogue molecule hydroxystilbamidine [10]. We conclude that for the homopolymer the binding behavior is influenced substantially by the ionic environment due to a strong salt-dependence of K_2^*. Thus the binding mode 2 is characterized by electrostatic interactions of the ligands to the duplex. The K_1-value varies only slightly with the salt concentration and hence this binding mode seems to depend mainly on non-electrostatic contributions.

Comparing the binding of berenil to poly (dA) · poly (dT) and to poly d(AT) the electrostatic contribution to the formation of complexes seems to be of major importance for the homopolymer, especially when small or moderate berenil concentrations are used. If the electrostatic interaction is accompanied by a release of counterions from the phosphate backbone, our interpretation should be underlined by a different thermodynamic, especially entropic profile of both polymers.

Similar to the reported data for netropsin binding to the two host duplexes [21] we calorimetrically obtained a mainly enthalpy-driven interaction ($\Delta H^0_{\text{bind}} = 28.8$ kJ/mol of drug) of berenil with poly d(AT) (Table 5). It is worth to note that this value is in fairly good agreement with the binding enthalpy of -36.8 kJ/mol of drug obtained by Zakrzewska et al. [36] using theoretical computations. The detected entropic contribution is relatively small and positive (~ 13–25 J/K mol), which was also observed for netropsin binding to the alternating copolymer [6,20,21,23]. However the binding of berenil to poly (dA) · poly (dT) is characterized by a smaller enthalpy contribution (-25.2 kJ/mol of drug) and a higher positive entropic term (~ 30–42 J/K mol). Breslauer et al. interpreted the positive entropic contribution as a release of bound water to the solvent and/or by fundamental differences in the hydration of both duplexes. In addition the release of ions to the solvent may also contribute to the positive entropy terms. This is in agreement with the interpretation of our binding data. However, compared to the difference in the entropic terms of poly (dA) · poly (dT) and poly d(AT) the binding enthalpies ΔH^0_{bind} are of similar magnitude. The high exothermic values of -28.8 kJ/mol and -25.2 kJ/mol can only be explained by the formation of strong hydrogen bonds between berenil and the polynucleotides.

The existence of electrostatic interactions was already assumed from the salt-dependence of the equilibrium constants for the two different binding modes. To evidence the existence of these electrostatic interactions and the release of counterions from the phosphate backbone further experiments were performed. At pH 7 berenil exhibits two positive charges and should, similar to other charged antibiotics, reveal a salt-dependence of K_{app}. To investigate these electrostatic interactions data evaluated by eq. (11) were analyzed (Fig. 4 and Table 4). The theory [16,17,29] predicts a straight line for a log–log plot with a slope of -1.76 and an intercept of $+0.4$ for a divalent cation. The detected slope for the binding to poly d(AT) of -0.92 indicates that on an average only one amidino-group seemed to be involved in the interaction.

From the Scatchard plots it was proposed that mainly the equilibrium constant K_2 of the binding mode 2 is influenced by the sodium concentration. Consequently berenil is partly bound to the polynucleotide by an interaction not involving the release of counterions to the solvent and thus the overall amount of ion-release is less than the theoretically predicted value of 2. As expected from the binding parameters and the higher positive entropy term the binding of berenil to poly (dA) · poly (dT) exhibits a larger negative value for the slope of a log–log plot than poly d(AT). We obtained a value of -1.22 which indicates that ~ 1.4 ions per drug molecule were released to the solvent. This result underlines the stronger electrostatic contribution to the binding mechanism for the homopolymer.

As described by Lohman et al. [16], the y-intercept of eq. (11) is related to the intrinsic free energy of the interaction of a ligand with one binding site. For a drug to base pair ratio of 0.33, $\Delta G^0 = -27.0$ kJ/mol of drug was obtained in the case of poly d(AT)–berenil complexes and -25.1 kJ/mol of drug for the homopolymer. These values are comparable to the experimentally determined free binding energies ΔG^0_{bind}. The large discrepancy of a factor >10 to the expected theoretical value -2.3 kJ/mol of drug might be attributed to site-specific interactions and/or to anion-release upon binding. To elucidate the effect of anions, experiments with [(n-butyl)$_4$NCl] were carried out as described above. The obtained slopes of -0.76 for poly (dA) · poly (dT) and -0.49 for poly d(AT), respectively (Fig. 4, Table 4) can not be explained by the increased ionic strength because the deviation from the theoretically predicted value of -0.24 is significant. Consequently anion-effects are supposed to be involved in the binding process.

In Tables 5 and 6 the calorimetrically determined van 't Hoff enthalpies ($\Delta H^{\text{DSC}}_{\text{v.H.}}$) and the binding enthalpies (ΔH^0_{bind}) are listed. The van 't Hoff enthalpy of the free poly d(AT) clearly shows a salt-dependence. The increase of the NaCl-concentration from 10 to 150 mM is accompanied by a decrease of the van 't Hoff enthalpy from $\Delta H^{\text{DSC}}_{\text{v.H.}} = -2790$ kJ/mol to $\Delta H^{\text{DSC}}_{\text{v.H.}} = -1701$ kJ/M. However, varying the salt concentration has no significant effect on the van 't Hoff enthalpy of poly (dA) · poly (dT) ($\Delta H^{\text{DSC}}_{\text{v.H.}} = -6240$ kJ/mol at 10 mM NaCl and $\Delta H^{\text{DSC}}_{\text{v.H.}} = -6700$ kJ/mol at 150 mM). The generally very high van 't Hoff enthalpies of poly (dA) · poly (dT) are emphasized because this indicates a highly cooperative "melting" of the base pairs.

Using the temperature-induced UV transition curves the $\Delta H^{\text{UV}}_{\text{v.H.}}$-values can be determined as introduced under materials and methods. For the free duplex these data are in fairly good agreement to the DSC-values (Tables 5 and 6). In Table 1 the $\Delta H^{\text{UV}}_{\text{v.H.}}$-values of the alternating polymer are listed for various salt concentrations, ranging from 10 mM to 250 mM NaCl. Table 2 shows the data for the homopolymer. In the case of the complexed polymers the van 't Hoff enthalpies could not be determined because the UV transition curves showed negative base lines. This base line effect was caused by the slightly temperature-dependent extinction coefficient of berenil resulting in unacceptable errors in the estimation of the slopes according to eq. (5).

From the observed van 't Hoff binding enthalpies and the ΔH^{DSC}-values the size of the cooperative unit σ^{DSC} can be estimated by the ratio $\Delta H^{\text{DSC}}_{\text{v.H.}}/\Delta H^{\text{DSC}}$. The obtained data are also listed in Table 5 and 6. At 10 mM NaCl and upon binding of berenil the length of the cooperative unit is substantially decreased from $\sigma^{\text{DSC}} = 92$ to $\sigma^{\text{DSC}} = 21$ for poly d(AT) and from $\sigma^{\text{DSC}} = 182$ to $\sigma^{\text{DSC}} = 23$ for poly (dA) · poly (dT). Obviously this effect is more pronounced for the homopolymer. This is the result of the helix-stabilization due to the binding of berenil, which is more effective in the poly (dA) · poly (dT)–berenil complex and thus prevents the duplex from "melting" cooperatively. From the similar σ^{DSC}-values for the complexes it is concluded that the size of the cooperative unit under low salt concentration is determined mainly by the binding of berenil. An analogous behavior was reported for the binding of netropsin to poly d(AT) by Marky et al. [20].

At a salt concentration of 150 mM NaCl the length of the cooperative unit for the free poly d(AT) and the analogue berenil complex is nearly identical ($\sigma^{\text{DSC}} = 57$ and $\sigma^{\text{DSC}} = 53$). This indicates that at high salt concentrations the cooperative length is mainly determined by the influence of the sodium ions and not by the drug molecule. It is noted that at the high sodium concentration the cooperative length of the free polynucleotide poly d(AT) is decreased by about 40 percent exhibiting the substantial influence of salt on the length of the cooperative unit.

Varying the NaCl-concentration induces a different effect on the homopolymeric nucleotide. At 150 mM NaCl a value of 192 for σ^{DSC} is calculated for the free duplex, which is identical to $\sigma^{\text{DSC}} = 182$ for the homopolymer at 10 mM NaCl, assuming an error of prediction of 10 percent. Hence, the cooperative length of poly (dA) ·

poly (dT) exhibits no salt-dependence. Taking this into consideration combined with the fact that berenil drastically influences the cooperative length at low salt concentration, a σ^{DSC}-value of ~23 is expected for the berenil complex at 150 mM NaCl. The calculated value of 38 shows that increasing the salt concentration does not influence the length of the cooperative unit in poly (dA) · poly (dT)–berenil complexes. The small increase of the σ^{DSC}-value from 23 to 38 is attributed to the smaller binding affinity of berenil at high ionic strength. Based on our results we conclude that the size of the cooperative unit of the alternating polymer poly d(AT) and the saturated complex depends seriously on the salt concentration. This might be a consequence of the relatively large width of the minor groove (12 Å). On the other hand poly (dA) · poly (dT) and the analogue berenil complex do not exhibit any significant salt-dependence of the cooperative length. This could be attributed to the intensive stacking of the adenine bases.

It is remarkable that in contrast to poly d(AT) at 150 mM NaCl the ΔH^0_{bind}-value of the poly (dA) · poly (dT)–berenil complex has changed significantly from −25.2 kJ/mol drug at 10 mM NaCl to −31.8 kJ/mol drug. Increasing the salt concentration resulted for complexes of berenil with both polymers in negligible entropy terms (Tables 5 and 6). Thus at 150 mM NaCl the binding reaction is mainly enthalpy-driven, whereas at 10 mM NaCl the entropic term significantly contributes to the binding process.

The combination of the thermodynamic data and the binding parameters obtained under different experimental conditions allows us to describe the overall binding behavior of berenil. For berenil binding to both model polymers at 10 mM NaCl hydrogen bonding seems to be the major driving force. Building up this hydrogen bonds may be favored slightly in the alternating polymer. For the homopolymer the lack of alternating purine–pyrimidine bases on opposite strands reduces the possibility to build up hydrogen bonds and consequently the observed absolute ΔH^0_{bind}-value is lower.

A further explanation for the difference in the ΔH^0_{bind}-values could be the different conformation of the two model duplexes. Theoretical computations for poly d(AT) [11,12] only suggest small distortions of the B-conformation upon binding of the ligand. Poly (dA) · poly (dT) usually adopts a heterogeneous, kinked B-conformation [1,33], which is also called B′-conformation. Pearl et al. [26] reported that berenil induces a larger distortion in poly (dA) · poly (dT) than in the case of the alternating sequence. If this conformational distortion is accompanied by a larger endothermic enthalpy contribution this may account for the small difference of 3.6 kJ/mol of the ΔH^0_{bind}-values between the model polymers.

Hence, from the comparison of the enthalpy data ΔH^0_{bind} berenil should bind preferentially to the alternating duplex. However, the larger positive entropic contribution changes the selectivity (+29.8 J/K mol, +41.6 J/K mol drug for poly (dA) · poly (dT); +12.7 J/K mol, 25.5 J/K mol drug for poly d(AT)) under conditions where the binding mode 2 dominates the association reaction. According to these experimental findings it is suggested that differences in the binding of berenil to the polymers poly (dA) · poly (dT) and poly d(AT) are mainly characterized by electrostatic contributions introduced by mode 2. These interactions depend strongly on the distance of the interacting components. The minor groove of the homopolymer in the B′-conformation has a size of 9.2 Å [11]. Possibly this smaller minor groove dimension compared with the 12 Å in poly d(AT) fits better to the shape of the small berenil molecule which is known to penetrate deeply into the minor groove. This fact would favor the electrostatic energy term upon binding of berenil to poly (dA) · poly (dT) compared to poly d(AT). It was reported that the electrostatic contribution to the overall binding energy is important in the case of charged ligands [12], which underlines our suggestion.

The calorimetric data and the binding parameters of berenil binding to poly (dA) · poly (dT) and to poly d(AT) complexes at 150 mM NaCl clearly demonstrate the influence of the ionic environment. Increasing the salt concentration to 150 mM NaCl changes the binding parameters drastically. Differences in the thermodynamic profile are also observed. Especially an entropic term is nearly absent for the binding reaction of berenil with the polymers under high salt concentrations. Such a striking effect can only be explained by the presence of electrostatic interactions.

5. Concluding remarks

The combination of spectroscopic and calorimetric data was used to characterize the binding of the drug berenil to the synthetic polynucleotides poly d(AT) and poly (dA) · poly (dT). The complex formation of both polymers with berenil is accompanied by a large exothermic enthalpy change, which is more pronounced for the alternating polymer poly d(AT). For both duplexes the large negative binding enthalpy is the major driving force for the association reaction. However, the entropy contributions are different for both polynucleotides. Berenil binds to poly (dA) · poly (dT) with a large and positive entropy change revealing the importance of electrostatic contributions to the binding process. The entropic term for of the association of poly d(AT) with berenil is also positive but smaller and thus electrostatic interactions are supposed to be of minor importance. A similar observation was reported for the binding of the drug netropsin to the synthetic polymers [6]. Our interpretation of the data is underlined by the fact that the association of berenil and poly (dA) · poly (dT) is followed by a release of 1.4-counterions to the solvent, whereas only a value of 1.0 was obtained for poly d(AT). Berenil binds to both model polymers with two distinct binding modes and reveals a site-size occupying ~ 3 base pairs at saturation. This binding behavior is similar to comparable non-intercalating compounds like DAPI and hydroxystilbamidine [10,18]. Only one binding mode which mainly contributes at low ligand concentrations to the overall binding behavior involves significant electrostatic interactions. This explains why the expected release of two ions to the solvent is not achieved. Summarizing the experimental data we note that a discussion of the selectivity of a drug binding with different interfering modes (mutual exclusion) to polymers is difficult because the variation of parameters like the ionic strength or the maintained drug to base pair ratio can substantially influence the complexation reactions.

The overall stability of the berenil complexes is attributed to the formation of hydrogen bonds. For one hydrogen bond Marky et al. [20] discussed a value of $\Delta H = -8$ kJ/mol to $\Delta H = -13$ kJ/mol, so that at least two hydrogen bonds seem to be necessary to understand the observed enthalpy of binding.

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