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The binding of Violamycin BI to poly-C

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Abstract

The binding constant K_{st} of Violamycin BI (VBI) to poly-C at small and medium values of the concentration ratio p (0–12) is determined using the procedure of Schwarz under two different conditions: at constant c°_{VBI} and variable c°_{P}, and at $p = c^{\circ}_{P}/c^{\circ}_{VBI}$ = const. The average value obtained for K_{st} is $3.3 \pm 0.1 \times 10^{4}$ M^{-1}, whereas the cooperativity parameter q of 13 characterizes a moderate cooperative interaction between adjacent bound ligands. In contrast, at large values of p (12–355) the formation of isolated bound dimers on the poly-C chain is observed. At pH 7, VBI dimerizes in solution with a dimerization constant strongly dependent on ionic strength: $K_d = 732 \pm 20$ M^{-1} and $(9.3 \pm 0.2) \times 10^{3}$ M^{-1} at $I = 0.02$ M and 0.2 M respectively. The lower and upper boundaries for the binding constant of the dimer to the polynucleotide at large values of p are 1.0×10^{-5} M^{-1} and 6.25×10^{-6} M^{-1} respectively.

Keywords: Cooperative binding; Stacking interaction; Antibiotic

1. Introduction

The interaction of small ligands with biopolymers remains a subject of interest in view of their possible role in biological function. The binding of organic dyes to linear polymeric chains often exhibits cooperative features. This can lead to concentration threshold effects. Equilibrium experiments can be evaluated by a model based on the linear Ising lattice of equivalent binding sites with nearest-neighbour cooperativity. The theory, developed by G. Schwarz, implies two types of intrinsic process: *nucleation* — the binding of an isolated ligand; and *aggregation* — the binding of the ligand in the immediate neighbourhood of one that is already bound [1–8]. Dourlent [9,10] extended the formalism of this theory to the case of two competitive binding sites for a small ligand, and applied it to proflavin interactions with a single-stranded polynucleotide poly-A and a double-helical structure DNA.

In the present paper, we report on the binding interactions of an antracycline antibiotic, Violamycin BI (VBI) with poly-C, adopting the basic model of Schwarz [4] with just one type of equivalent binding site. This restricts cooperative interactions to those with nearest neighbours.

2. Materials and methods

Violamycin BI, isolated from fermentation cultures of *Streptomyces violaceus*, was provided by

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Dr. G. Strauss [11]. VBI solutions were freshly prepared in a phosphate–EDTA buffer at pH 7, and kept in the dark before each measurement to avoid photobleaching. The concentrations were determined by measuring the absorption at $\lambda = 500$ nm using $\varepsilon_{500} = 10.250\ M^{-1}\ cm^{-1}$ [12].

Poly-C, polycytidilic acid (5′), was purchased as the potassium salt from Sigma. Solutions of poly-C were prepared in the same buffer and their concentrations determined by measuring the absorbance at $\lambda = 270$ nm, $\varepsilon_{270} = 6.300\ M^{-1}\ cm^{-1}$ [13]. All experiments were carried out at $I = 0.02$ M, the natural ionic strength of the buffer, without adding any other salt. For the absorbance measurements we used a Hewlett Packard 9153C spectrophotometer and a Cary 2200. To obtain the difference spectra for spectrophotometric titration we used two cells ($\ell = 1$ cm) in each cell compartment as follows: the first cells initially contained a VBI solution of 3.9×10^{-5} M and the second cells buffer. The volume of solution each of the four cells was 2.2 ml. In the sample path, we added a mixture of poly-C (8.9×10^{-4} M(P)) and VBI (3.9×10^{-5} M) to the cell containing VBI. In the reference path, we added the same volume of poly-C (8.9×10^{-4} M(P)) to the cell containing buffer. We repeated this addition several times cumulatively, and each time obtained the difference spectrum and the differences ΔA at various wavelengths. All the spectrophotometric measurements were carried out at 20.5°C.

5′CMP (cytidyl monophosphate) disodium salt was also obtained from Sigma, and used in the same buffer solution to check the possible interaction of VBI with the bases of poly-C. CD spectra were measured using the Spectropolarimeter J-720 from JASCO.

3. Results

3.1. The dimerization of free Violamycin BI

To evaluate the influence of self-dimerization on the binding of this ligand with poly-C under the above-mentioned conditions, the dimerization constant K_d of free VBI must be known at pH 7 and $I = 0.02$ M. In order to determine this parameter, we carried out spectrophotometric measurements on dif-

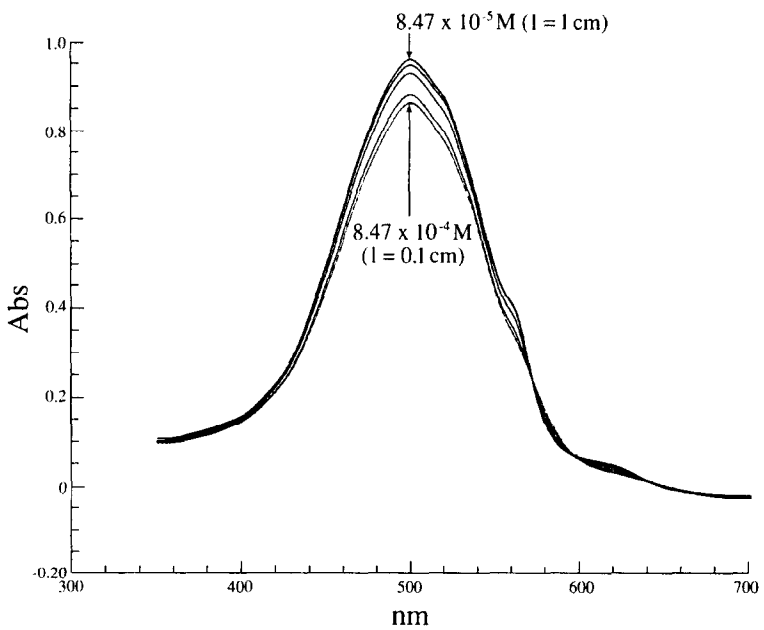

Fig. 1. Absorbance of solutions of VBI with increasing concentration and corresponding decreasing length of the optical path such that $c\ell$ = const. Dimer formation is indicated by hypochromic changes and by the appearance of isobestic points.

ferent concentrations of VBI, prepared from the same stock (8.47×10^{-4} M) at $c\ell = 8.47 \times 10^{-5}$ M cm, and obtained the spectra with three isosbestic points as shown in Fig. 1.

Using the absorbances at $\lambda = 500$, nm we proceeded as described elsewhere [12]. We obtained from the plots (with a linear regression coefficient $r = \pm 0.955$) a value of $\varepsilon_d^{500} = 8363 \pm 200$ M^{-1} cm^{-1} for the molar absorption coefficient of the dimer. This result agrees within experimental error with the value 8500 M^{-1} cm^{-1} obtained previously at $I = 0.2$ M [12]. This shows that the molar absorption coefficient is not dependent on the ionic strength. From the same plot, the dimerization constant $K_d = 732$ M^{-1} is found to be more than ten times lower than the value of 9300 M^{-1} obtained at $I = 0.2$ M. The higher ionic strength favours the dimerization process, since it lowers the electrostatic repulsion between the charged dimerizing molecules. At pH 7 each monomer carries two positive charges [14].

3.2. Cooperative binding at small and medium polymer-to-ligand ratios

Experiments were carried out at constant total VBI concentration with differing amounts of poly-C, varying the ratio $p = c_P^\circ / c_{VBI}^\circ$. c_P° is expressed as molarities of the nucleotide subunits. At low p the formation of VBI aggregates on the polymer chain is favoured.

The decreasing absorbance of the free VBI at $\lambda = 500$ nm is associated with the appearance of three isosbestic points. This is very similar to the spectrum obtained by free dimerization at larger concentrations of VBI. It should be noted that only the isosbestic point situated at the longest wavelength undergoes a significant shift (≈ 20 nm). This behaviour is characteristic of an equilibrium between the free ligand (VBI) and the stacked complex (VBI–poly-C). The metachromatic effect observed in the absorption band of VBI may be attributed to the interaction of the bound VBI molecules with each other along the poly-C, acting primarily as a template supplying negative charges to neutralize the repulsive forces between the positively-charged VBI molecules. To evaluate our experimental data, we adopted the treatment of Schwarz by plotting γ^* versus p, γ^* being the total fraction of free ligand (monomers and possibly dimers):

$$\gamma^* = \gamma(1 + 2K_d c_{VBI}^\circ \gamma) \quad (1)$$

where K_d is the previously determined dimerization constant (see Section 3.1) and γ is the fraction of free monomeric VBI. At the concentrations used in these experiments the corrections for free dimerization always remained below a few percent.

To compute $\gamma = c_{VBI(monomer)}^{free} / c_{VBI}^\circ$ from the experimental absorbances, it was necessary to investigate this system at a constant p value, but variable VBI and poly-C concentrations. The equation

$$\gamma = \frac{\varepsilon_{app} - \varepsilon_{st}}{\varepsilon_{VBI} - \varepsilon_{st}} \quad (2)$$

is used to obtain the fraction of free monomeric ligand. This equation is valid if the concentration of monomeric bound ligand is negligible, and if the concentration of free dimer is also negligible or if the extinction coefficient of the ligand in the free dimer is nearly equal to that of stacked ligand. To use this equation, the extinction coefficient of free monomeric ligand ϵ_{VBI} and that of bound and stacked ligand ϵ_{st} must be known. ϵ_{VBI} has been found from previous measurements on solutions without poly-C [12]. ϵ_{st} is obtained from a series of measurements with increasing c_{VBI}° and c_P° at constant ratio $p = 7.93$. For moderate values of $gp > 1$ (g is the number of ligands that can be accommodated on a single polymer segment), such that an excess of binding sites is always present, and under conditions of cooperativity, the following relation is valid for values of $K_{st} c_{VBI}^\circ > 1$:

$$\varepsilon_{app} = \varepsilon_{st} + (\varepsilon_{VBI} - \varepsilon_{st}) \frac{1}{K_{st} c_{VBI}^\circ} \quad (3)$$

K_{st} is the equilibrium constant for extending an already existing stack of cooperatively bound ligands by an additional molecule, or for cooperative binding of a ligand to a neighbouring bound molecule. Values of ε_{st} and K_{st} are obtained from a plot of ε_{app} versus $1/c_{VBI}^\circ$.

The predicted linear relationship is obtained at least for smaller values of $1/c_{VBI}^0$. With increasing c_{VBI}^0, greater and greater fractions of VBI will be bound and, hence, it is found that $\varepsilon_{app} \rightarrow \varepsilon_{st}$ on

extrapolating $1/c^{\circ}_{VBI} \rightarrow 0$. The molar absorption coefficient of the stacked complex ε_{st} is given by the intercept on the ordinate axis, and is found to be $\varepsilon_{st} = 8000 \pm 200\ M^{-1}\ cm^{-1}$. An estimate of $1/K_{st}$ is also obtained from the slope, giving $K_{st} = 3.2 \times 10^4\ M^{-1}$ as shown in Fig. 2.

From Eq. (2), as ε_{st} is known, we can calculate γ_{VBI} values, which are required to compute γ^* from Eq. (1). The plot of γ^*_{VBI} versus p is shown in Fig. 3. After a rapid decrease in the range of small p, γ^* levels off to a much slower decrease above $p \approx 2$. This suggests a cooperative binding of VBI to poly-C. The bound fraction of the ligand is described by the equation:

$$\theta gp = 1 - \gamma^* \qquad (4)$$

where θ is the fraction of binding sites occupied by the ligand, also called the degree of saturation. At first, when $gp \ll 1$, all the binding sites are occupied. The degree of saturation θ remains equal to unity in this region and γ^* will be proportional to p. By extrapolating this linear part to the abscissa, one obtains from the intercept for which $pg = 1$ (or from its slope) the value of g, the number of binding sites per monomeric segment of the polymer. We found $g \simeq 0.43$, at first sight a plausible value, because VBI has two positive charges and the monomeric segment of poly-C, containing a phosphate group,

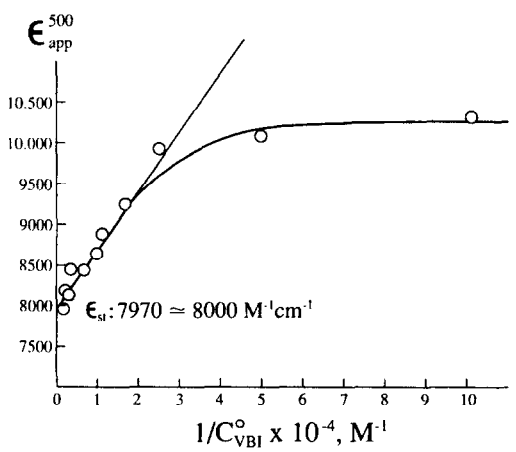

Fig. 2. ε_{app} versus $1/c^{\circ}_{VBI}$ at constant $p = 7.93$. At values of c°_{VBI} below 1×10^{-5} M there is practically no binding. Binding sets in at $c^{\circ}_{VBI} \simeq 2.5 \times 10^{-5}$ M and saturation begins when $c^{\circ}_{VBI} > 5 \times 10^{-4}$ M. The apparent binding constant, estimated from the slope, is $K_{st} = 3.2 \pm 0.7 \times 10^4\ M^{-1}$.

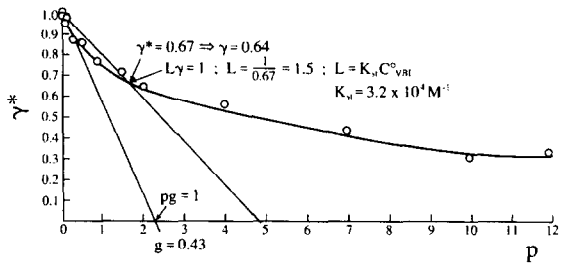

Fig. 3. Titration curve of Violamycin BI at constant concentration $c^{\circ}_{VBI} = 4.63 \times 10^{-5}$ M with increasing concentration of binding sites. The intersection of the extrapolated initial slope with the abscissa yields the average number of binding sites occupied by a ligand molecule. A line with half this slope allows determination of the binding constant K_{st} (see text).

offers only one negative charge. It should be mentioned that the concentration of the monomeric segments defines the polymer concentration c°_{P}. Thus, with g binding sites per segment, the total concentration of binding sites is gc°_{P}. We also tested this behaviour at a lower concentration of VBI (3.9×10^{-5} M) and found the same value of g.

Once g is known, a straight line of slope $-g/2$ can be drawn, as shown in Fig. 3. This intersects the binding curve at $\gamma^*_{VBI} = 0.7$. For the point of intersection, the theory shows [3] that $s = \gamma L$ becomes equal to unity, where s is a dimensionless parameter and L is the binding strength, a quantity which is defined as the product of the total ligand concentration and the binding constant:

$$L = K_{st} c^{\circ}_{VBI} \qquad (5)$$

With $L = 1/\gamma_{s=1} = 1.5$ at the intersection point, the binding constant K_{st} of the stacked complex can immediately be evaluated:

$$K_{st} = \frac{1.5}{4.63 \times 10^{-5}\ M} = 3.2 \times 10^4\ M^{-1}$$

The theory shows also that if γ^*_{VBI} is expressed as

$$\gamma^*_{VBI} = \frac{s}{K_{st} c^{\circ}_{VBI}} \left(1 + 2\frac{K_d}{K_{st}} s\right) \qquad (6)$$

K_{st} can also be computed by inserting the special value of γ^*_{VBI} at the intersection point for which $s = 1$. This gives $K_{st} = 3.2 \times 10^4\ M^{-1}$, which is in very good agreement with the initial value. We confirmed these values with another method, by

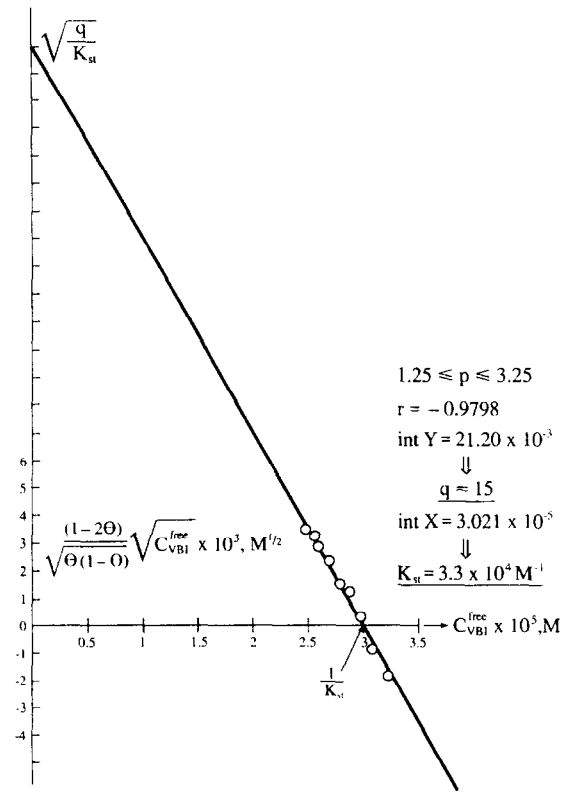

Fig. 4. Determination of the binding constant K_{st} from experimental points on Fig. 3.

plotting $(1-2\theta)\sqrt{c_{VBI}}/\sqrt{\theta(1-\theta)}$ versus c_{VBI}, the free monomeric Violamycin concentration, according to the equation:

$$\frac{(1-2\theta)\sqrt{c_{VBI}}}{\sqrt{\theta(1-\theta)}} = \sqrt{\frac{q}{K_{st}}} - c_{VBI}\sqrt{qK_{st}} \qquad (7)$$

A straight line with two intercepts gives $\sqrt{q/K_{st}}$ and $1/K_{st}$ respectively, as Fig. 4 shows. We computed the values of θ using Eq. (4).

Our experimental data (with a linear regression coefficient $r = -0.9738$) gave from the intercept on the abscissa $K_{st} = 3.3 \times 10^4\ M^{-1}$, in agreement with the above-mentioned values. We consider this last method of determination of the binding constant K_{st} to be more accurate, because both Eq. (5) and Eq. (6) use the approximate intersection point of the straight line with the binding curve.

Another confirmation of the K_{st} value resulted from the difference spectra presented in Fig. 5. Of note are the isobestic points at ≈ 572 nm, ≈ 600 nm and ≈ 660 nm. The first point coincides with the corresponding isobestic point of the free dimer spectrum (Fig. 1), whereas the second is shifted by about 7 nm and the third by about 20 nm to longer wavelengths. Concomitant with the plot of the differ-

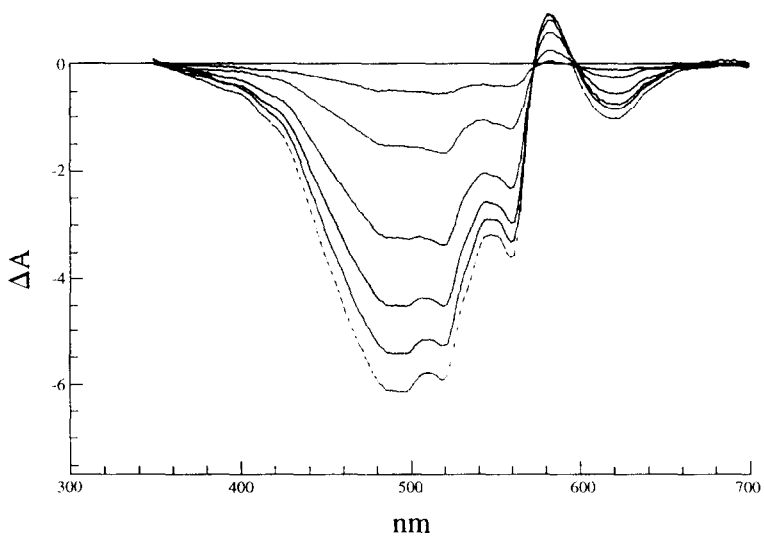

Fig. 5. Difference spectra carried out with two cells in each compartment, such that the increasing concentration of poly-C in the sample solution is compensated for in the reference path, and the concentration of VBI remains constant at $c^{\circ}_{VBI} = 3.90 \times 10^{-5}$ M.

Table 1
Computed data from the measurements of difference spectra, $c^{\circ}_{VBI} = 3.90 \times 10^{-5}$ M

V_{ad} poly-C (ml)	$c^{\circ}_{P} \times 10^{5}$ (M(P))	$c^{\circ}_{P} c^{\circ}_{VBI} \times 10^{10}$ (M^2)	$(c^{\circ}_{P} + c^{\circ}_{VBI}) \times 10^{5}$ (M)	$\Delta A_{500} \ell = 1$ (cm)	$\frac{c^{\circ}_{P} c^{\circ}_{VBI}}{\Delta A_{500}} \times 10^{8}$ (M^2 cm)
0.01	0.393	1.53	4.29	−0.0052	−2.95
0.05	2.310	8.97	6.21	−0.0156	−5.75
0.10	5.88	22.9	9.78	−0.0325	−7.05
0.20	12.2	47.5	16.1	−0.0449	−10.6
0.30	20.0	78.0	23.9	−0.0538	−14.5
0.40	28.2	110	32.1	−0.0606	−18.2

ence spectra, the printed ΔA values are presented in Table 1 for $\lambda = 500$ nm. We used Eq. (8), the exact variant of the Hildebrand–Benesi equation for the case when absorbance A_{∞} is unknown at the end of the titration and when neither of the reacting partners is in excess:

$$\frac{c^{\circ}_{P} c^{\circ}_{VBI}}{\Delta A} = \frac{1}{K_{st} \Delta \varepsilon} + \frac{1}{\Delta \varepsilon}(c^{\circ}_{P} + c^{\circ}_{VBI}) - \frac{\Delta A}{(\Delta \varepsilon)^2} \quad (8)$$

Here, $\Delta A = A - \varepsilon_{VBI} c^{\circ}_{VBI}$ for $\ell = 1$ cm and $\Delta \varepsilon = \varepsilon_{st} - \varepsilon_{VBI}$. Evaluation by least-squares parameter fitting of the data in Table 1 gives $\Delta \epsilon = -1900$ and $K_{st} = 3.2 \pm 0.7 \times 10^{4}$ M^{-1}. The $\Delta \epsilon$ value obtained in this manner is smaller than the difference −2250 obtained from independent evaluations of ε_{st} and ε_{m}, but the value of K_{st} is consistent with those obtained by other methods. The Benesi–Hildebrandt treatment is not truly applicable in the case of cooperative binding, since it is based on a simple bimolecular equilibrium between a ligand and independent binding sites. Therefore, it gives no clue as to the cooperativity parameter q.

The cooperativity parameter q, which measures the extent of cooperative interaction between nearest neighbours, can be computed from the intercept on the ordinate of Fig. 4, giving $q = 15$.

This parameter may also be computed by other methods according to the treatment of Schwarz [3], e.g. using the following equation:

$$\frac{s}{(1-s)^2} = q \frac{\theta(1-\theta)}{(1-2\theta)^2} \quad (9)$$

This equation represents a straight line passing through the origin. In our case, the experimental data from Fig. 6 led to a slope with $q = 11$ and a linear regression coefficient of $r = 0.994$. The values of s are obtained from $s = L\gamma = K_{st} c^{free}_{VBI}$ with $L = 1.5$ as indicated above, and θ as defined in Eq. (4). We shall use 13 as the average value of q.

Knowledge of q permits computation of the parameters describing the cooperativity of VBI binding to poly-C, e.g. the energy decrease, the average number of VBI ligands in an uninterrupted VBI sequence on the poly-C chain (or of the unoccupied gaps between these clusters), and also of the binding

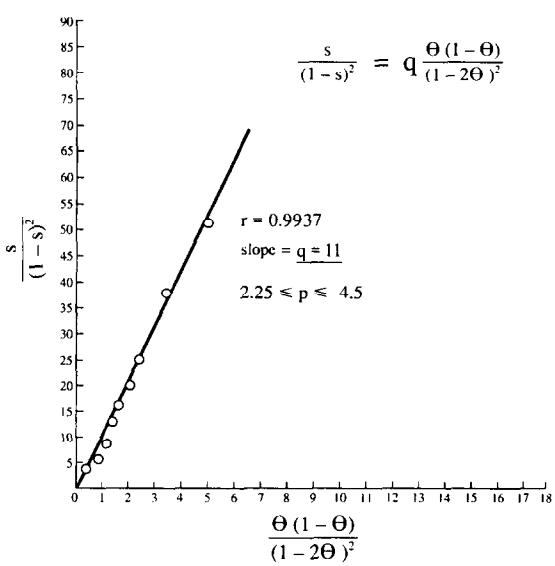

Fig. 6. Determination of the cooperativity parameter q according to Eq. (9).

constant of an isolated bound VBI (unstacked VBI). However, at high values of p it is not possible to neglect the contribution of non-cooperatively bound ligand in Eq. (2).

The free enthalpy decrease due to the cooperative interaction of the nearest-neighbour ligands on the polymer is:

$$RT \ln q = -\Delta G^{\circ}_{coop} \quad (10)$$

giving $\Delta G^{\circ}_{coop} = -6.4$ kJ mol^{-1}. The average number m_* of ligands in an uninterrupted VBI sequence derived for infinite chain length can be computed from

$$m_* = \sqrt{q}\,\frac{\lambda_0}{\sqrt{s}}\sqrt{\frac{\theta}{1-\theta}} \quad (11)$$

in which the parameter λ_0 is given by

$$\lambda_0 = 1 + \sqrt{\frac{s}{q}}\sqrt{\frac{\theta}{1-\theta}} > 1 \quad (12)$$

If we take into account the behaviour of the binding curve (Fig. 3) at the point where it is still subject to a fairly sharp transition around $s = 1$, where θ always equals 1/2, and substitute these values into Eq. (12), $\lambda_0 = 1 + \sqrt{1/q} = 1.2774$ is obtained. Under these conditions ($s = 1$, $\theta = 1/2$), Eq. (11) becomes:

$$m_* = \sqrt{q}\,\lambda_0 \quad (13)$$

and, therefore, the average stack length m_* is about five VBI molecules when the polymer is half covered with the ligand.

The degree of cooperativity is determined by the ratio of the binding constant K_{st} for the stacked ligand to that of the unstacked ligand K:

$$q = K_{st}/K$$

If accurate values for K_{st} as well as for q are available, the equilibrium constant K of the nucleation process can be calculated immediately. In our case, $K = 2500$ M^{-1}.

3.3. Dimer binding at large p-values

We have attempted to confirm the results obtained so far by extending the measurements to high ratios $p = c^{\circ}_P/c^{\circ}_{VBI}$. The behaviour of solutions at constant c°_{VBI} and with increasing c°_P is shown in Fig. 7. Above $p \simeq 30$, the depression of the apparent extinction coefficient due to a stacking interaction is re-

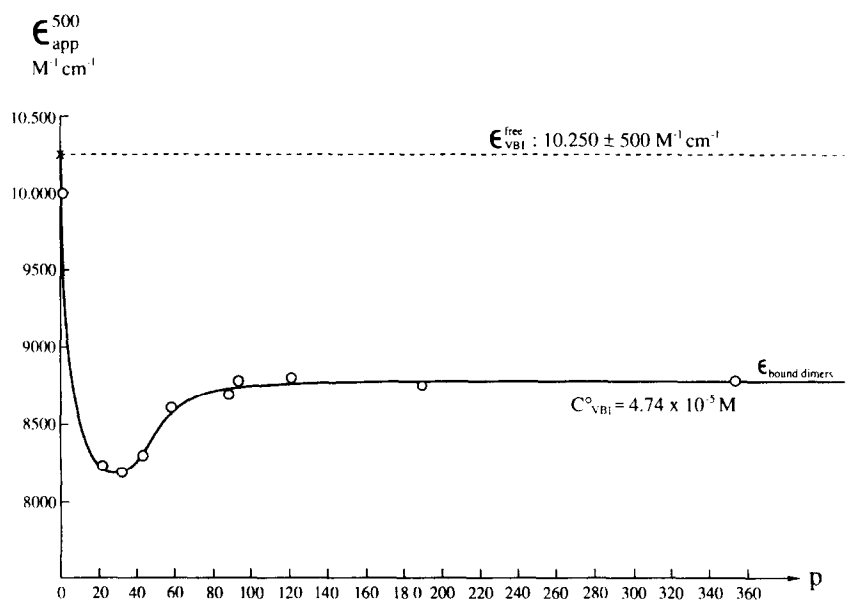

Fig. 7. The apparent extinction coefficient of VBI in solutions with $c^{\circ}_{VBI} = 4.74 \times 10^{-5}$ M and increasing poly-C concentration remaining below the extinction coefficient of free VBI, indicating dimer binding at a high excess of poly-C.

laxed when more and more binding sites become available for binding. The corresponding increase in the apparent extinction coefficient does not continue, however, until it reaches the value of isolated (unstacked) bound ligand (expected to be about the same as that of free, unbound and non-dimerized VBI). Instead, it levels off at $p \approx 90$ and then remains constant, even at a very high excess of binding sites on the polymer. This behaviour precludes the application of a procedure that allows an alternative determination [7,8] of the cooperativity parameter q. The method relies on the fact that the initial and final slopes of the optical absorption of solutions with increasing p and c°_{VBI} are described by parallel lines in a plot of A versus c°_{VBI}. This is not the case if the ligand is not bound at independent isolated binding sites with an extinction coefficient equal to that of the free monomeric ligand.

We have tested whether an additional non-cooperative interaction between bound VBI and the cytidine bases of the polymer subunits could be responsible for the lowering of the extinction coefficient at high p-values. Such behaviour has been observed for some dyes in the presence of poly-A [15,16]. For this purpose, we tested cytidine in large excess over VBI as well as 5′CMP up to $p = 300$. The latter experiments were carried out under conditions in which the system is well defined [17], namely at pH 7.1. This is above the value of 6.3, at which 5′CMP has two negative charges, and below 8.2, the pK_a of VBI protonation. No change was observed in the spectrum of the sample to which 5′CMP was added, in comparison to the spectrum of lone VBI at the same concentration. An interaction between bound VBI and the cytidine bases of poly-C, leading to the observed levelling off of ε_{app} at high p-values, can thus be excluded. If an interaction exists, it does not lead to spectral changes.

A mutual interaction between the ligand chromophores in a bound dimer is also indicated by the findings of Löber et al. [14], who studied the binding of VBI to polyphosphate and to DNA. At $c^{\circ}_{VBI} = 3.0 \times 10^{-6}$ M, the fluorescence intensity in the presence of polyphosphate at low p drops strongly to about one tenth of that of a polyphosphate-free solution, and remains depressed even at $p = 300$, although in this case the influence of chromophoric residues on the polyionic polymer chain is neglected. In the case

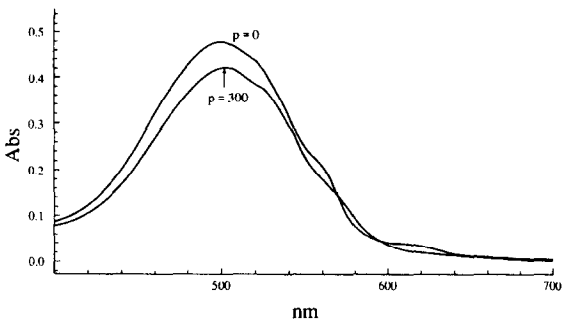

Fig. 8. The absorption spectrum of bound VBI at a high excess of poly-C, similar to the spectrum of the free dimer in Fig. 1.

of DNA-binding, the authors conclude that VBI intercalates with the DNA-bases. This conclusion is supported by the observed spectral changes and by the fact that about 6.4 nucleotide phosphates are required for the binding of one VBI molecule at saturation. The apparent binding constant of VBI to DNA is $(4.8 \pm 0.3) \times 10^{-7}$ M^{-1} at $I = 0.1$ M, indicating a strong affinity to DNA of VBI as an intercalator. The absence of a specific influence of the cytidilic residues of poly-C suggests that the driving forces for intercalation in DNA are not primarily due to specific interactions with the bases, but to a configurational entropy gain of the solvent structure when the VBI-chromophore is inserted in the base-stack of the DNA molecule.

The spectrum of bound VBI at large p is different from that of free VBI, as shown in Fig. 8. It also differs from the spectrum of stacked VBI at low p, where the isobestic points are shifted to a different position. However, it is very similar to the spectrum of the free VBI dimer. This leads to the conclusion that at high p-values VBI is predominantly bound in a dimeric state to poly-C.

4. Discussion

The low value of the cooperativity parameter q is not easily reconciled with the persistent existence of bound dimeric entities at a high excess of binding states. It can only be understood if the binding mode and the type of interaction between the neighbouring ligands at low values of p (at which there is competition for binding sites) is different from that at a

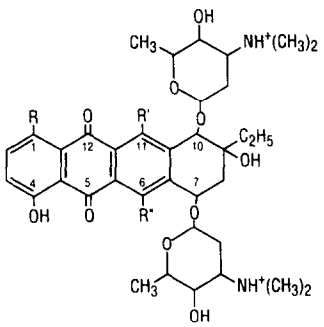

Fig. 9. Chemical structure of Violamycin BI [14]. The compound used contains OH-substitutions at two or all of the carbon atoms 1, 6 and 11.

high excess of binding sites. It is important to realize that VBI is a rather large molecule. The purified compound contains several related chemical structures, differing with respect to hydroxy substitutions on the anthrachinone skeleton of the chromophoric part of the molecule. The structure is shown in Fig. 9, based on the information given in Ref. [14]. The distance between the two positively-charged centres is about 12 Å when the two glucosyl residues are extended outwards, whereas the distance between the two consecutive negative centres on the phosphate backbone of poly-C is only about 6 Å. These distances were obtained from geometrically optimized models using the computer program HYPERCHEM. A stacked form in which both positive charges of VBI neutralize two neighbouring negative charges of the poly-C backbone is difficult to visualize. The value of $g = 0.43$ at low p, indicating that a bound ligand in the stacked form occupies about two polymeric sites on the backbone, can therefore hardly be interpreted in this way. A structure in which the VBI molecules form a staggered stack, with their anthracycline moieties superposed in a staircase-like pattern, leading to two helical arrangements of positively-charged glucosylic residues on the outside, stabilized by the negative charges of a poly-C chain, located between the two helices, is a possible alternative. Such a structure should reveal itself by circular dichroism. This is indeed the case, as shown in Fig. 10. The positive circular dichroism of free VBI follows its absorption spectrum with a maximum at 500 nm. At $p = 4$, at which stacks of several VBI molecules are formed upon binding to poly-C, the CD-band of VBI is enhanced, and a negative CD-band with a maximum at about 560 nm is superimposed. The enhanced circular dichroism remains when VBI binds as dimers at a high excess of binding sites $p = 250$, but the superimposed negative band is significantly weakened.

The rather low value of the cooperativity parameter q may indicate that the stacked structure involves less stable conformations or orientations of the glycosylic parts of the molecule. The large value of the nucleation equilibrium constant $K_{nucl} = K_{st}/q = 2500$, corresponding to a standard free energy of $\Delta G^{\circ}_{nucl} = -19.5$ kJ mol^{-1} (assuming the usual 1 M standard state definition extrapolated from infinite dilution for dissolved ligand and binding sites in a solvent of given ionic strength), is too large to be entirely due to the partial neutralization of two pairs of opposite charges in an aqueous medium. The decrease in electrostatic energy when two opposite charges are brought together from infinity to a distance of 3 Å in a continuum with the dielectric

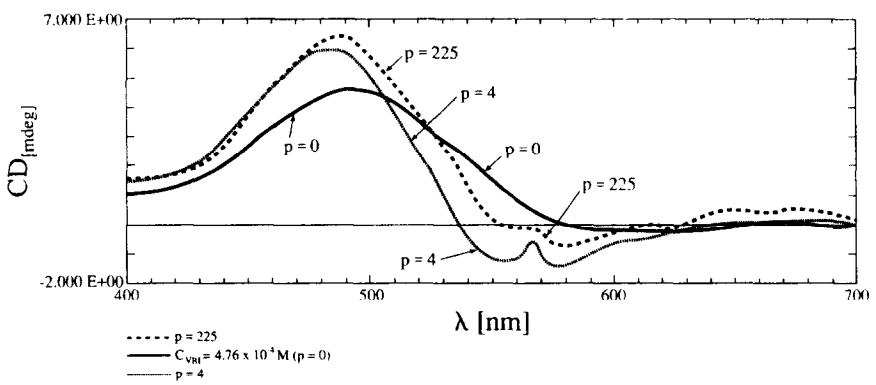

Fig. 10. CD spectra of free VBI ($p = 0$) and VBI–poly-C ($p = 4$ and $p = 225$) at the same concentration $c^{\circ}_{VBI} = 4.76 \times 10^{-4}$ M.

constant of water is only -6 kJ mol^{-1}. At the ionic strength used in the experiments, the value is even smaller. Additional factors, increasing the stability of an (isolated) bound molecule of VBI to poly-C, such as changes in solvation and hydrogen bonding [18], must be invoked to explain the strong binding. The entropy loss involved in the binding must also be accounted for. The changes in solvation around the hydrophobic part of the molecule, however, usually overcompensate this and contribute to the stability.

The persistent existence of bound dimers at high values of p must be due to a stable arrangement of two neighbouring ligands which is different from the stacked structure at low p. The spectrum shows that this arrangement must be very similar to that of the free dimer. The electrostatically most stable complex of two VBI molecules in the free dimer consists of an antiparallel arrangement of the overlapping chromophores. Such a structure can also form on the poly-C chain, but it probably covers a longer segment on the chain than two molecules in the stacked conformation at low p. This would explain the preference for the stacked conformation when there is competition for binding sites.

It was not possible to determine experimentally the equilibrium constant for dimeric binding at large p-values. Using the measured values of K_d, K_{st} and q, an estimate is given by

$$K_{db} = K_{st}^2/qK_d$$

giving $K_{db} = 1.076 \times 10^5$ M^{-1} for the reaction

$$uuuu + V_2 \rightleftharpoons uaau$$

where *uuuu* is a sequence of unoccupied binding sites and V_2 represents the free dimer. This estimate is only a lower boundary, because, as indicated, the number of monomeric units of poly-C per binding site for a bound VBI dimer at large p may be larger than four, which is the number for the stacked form at low p where the values of K_{st} and q apply. The binding may be stronger when more monomeric units are involved. The geometric arrangement of the charges may be more favourable and the hydrophobic bonding forces may also be enhanced. An upper boundary for K_{db} is given by

$$K_{db} = K_{nucl}^2$$

giving $K_{db} = 6.25 \times 10^6$ M^{-1}. This value would apply if each molecule of the dimeric pair interacted with the polymer in the same way as an isolated molecule.

It is evident that the binding of VBI to poly-C is more complicated than represented by the simple model on which the theory used in our evaluations is based. This model accounts for only one kind of neighbour interaction, independent of the length of a stack of interacting molecules. Nevertheless, the analysis of our experiments with this model allows characterization of the nature of the cooperativity in the interactions between rather large, multiply-charged molecules with a hydrophobic chromophore. Without a neutralizing polymer present, their tendency to form dimers is very strongly dependent on the presence of a shielding atmosphere of counterions at sufficient ionic strength. The presence of an oppositely-charged polymer stabilizes the interactions of dimers or of a multimeric stack of molecules. When the long-range electrostatic repulsion forces are sufficiently weakened by the attraction to the oppositely-charged polymer, the short-range van der Waals attraction forces provide the required stability for the multimeric structure. This situation is somewhat comparable to the formation of the nucleosome, which is also stabilized by the wrapping of the oppositely-charged DNA-polymer. In the case of VBI, the multimeric structure competes with the formation of bound dimers, which are the most stable form when a large excess of binding sites is available on the polymer.

5. Unlinked References

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