Biophysical Chemistry, 47 (1993) 277–283
Elsevier Science Publishers B.V., Amsterdam

BPC 01781

Self-association of violamycin (VBI)

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(Received 18 December 1992; accepted in revised form 3 May 1993)

Abstract

Violamycin (VBI) self-associates in aqueous solution at concentration greater than 7×10^{-5} *M*. We report in this paper visible spectrophotometric and kinetic experiments that characterize this self-association. We started from a simple dimerization model supported by our experimental data, and we used different methods described in literature for the computation of the dimeric extinction coefficient ε_d at various wavelengths and the dimerization constant $K_d = 9300\ M^{-1}$.

Keywords: Violamycin; Self-association; Spectrophotometry; Temperature jump; Anthracycline; Dimerization

1. Introduction

Violamycin (VBI) is an anthracycline antibiotic isolated by Fleck, Strauss et al. [1] from fermentation cultures of *Streptomyces violaceus*, strain IMET 6844. Löber et al. [2] described for the first time its absorption spectrum determined by the aglycone moiety and reported for the molar absorption coefficient in neutral unbuffered aqueous solution a value $\varepsilon = 12000\ M^{-1}$ at 500 nm, VBI's absorption maximum. They did not find changes in absorption spectra within the concentration range 10^{-6}–10^{-3} *M*, which could account for VBI aggregation. Many years later Löber et al. [3] published the VBI structure, its average molecular weight (779) and another value for ε_{500} i.e. 9750 M^{-1}, in aqueous solution at neutral pH. They observed that the compound obtained by the usual preparation is not pure. Two substituents on the anthracycline chromophore part of the molecule may be either H or OH, leading to a mixture of three related compounds which are present in approximately equal amounts. At the same time they specify that VBI self-associates in aqueous solution at concentration greater than 5×10^{-4} *M*.

The reported modified values of molar coefficient, and of the concentration range without aggregation processes, determined us to reinvestigate VBI in neutral buffered aqueous solution.

The knowledge of basic spectroscopic properties of VBI can be exploited in investigation of its interaction with biological macromolecules. This interaction competes with the equilibrium of

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self-association, so it is important to study the latter separately.

2. Materials and methods

Violamycin BI, isolated and purified at the Central Institute of Microbiology and Experimental Therapy, Jena, was offered by Dr. D.G. Strauss. VBI solutions were prepared in a phosphate–EDTA buffer, pH 7, ionic strength $I = 0.2$ M (Na^+) using analytical grade reagents (Merck).

Spectrophotometric titrations were carried out in a quartz cell of 1 cm pathlength, containing 2.5 ml of a dilute VBI solution (1×10^{-6} M), by adding various volumes of a concentrated VBI solution (3.16×10^{-3} M) with microliter pipettes (Pedersen, Denmark). The absorbance measured at $\lambda = 500$ nm corresponding to the calculated concentration at each addition, permitted the computation of the apparent molar absorption coefficient ε_{app} and therefore ε_m of the monomeric VBI. We estimated also the concentration range of VBI solutions without aggregation processes.

Visible absorption spectra were also recorded at constant $c \times l$, using various concentrations of VBI solutions in quartz cells of 0.1, 0.2, 0.5, 1.2 and 4 cm pathlength l, to determine the equilibrium constant of the dimerization process K_d at 20.5°C. These spectrophotometric measurements were carried out with a Specord M400 Jena, UV–Vis spectrophotometer.

For the calculation of K_d, ε_m, ε_d parameters from different linear plots, we used a Texas Instruments TI-66 with a linear regression program which offers the values of slope, intercept and regression coefficient. At the same time in order to obtain the above three parameters, we used routine DBCONF from IMSL, MATH HBRFRI program which minimizes a function of N variables (in our case, only three) subject to bounds on the variables, using a quasi-Newtonian method and a finite-difference gradient with the option that all variables are non-negative.

For our experiments we used also the temperature-jump technique to determine the dimerization equilibrium constant K_d from kinetic measurements of the rate constants of dimer formation k_{12} and of its dissociation k_{21}.

3. Results and discussion

3.1. Spectrophotometric titration

The first indication of violamycin self-association is obtained from the spectrometric titration data, illustrated in Fig. 1.

Over the range 10^{-6}–10^{-4} M, ε_{app} seems to be constant at 10250 M^{-1} cm^{-1}, the molar absorption coefficient of the monomeric VBI at $\lambda = 500$ nm. From other spectrophotometric titrations we observed that Beer's law is verified only to a concentration of $\sim 7.7 \times 10^{-5}$ M, that is to a lower concentration of VBI than that reported in the literature.

From the slope of the linear plot one obtains an ε_m value practically the same within our experimental errors 10250 ± 500 M^{-1} cm^{-1}. On the other hand, Löber remarked — and we observed it to — that the increase of VBI total concentration is not accompanied by a shift of the maximum, in the investigated concentration range, in contrast to many other dyes which self-associate.

3.2. Spectrophotometric measurements at constant $c \times l$

In Fig. 2 one notices the decreasing of absorbance with the increase of VBI concentration while keeping the product $c \times l$ constant at a

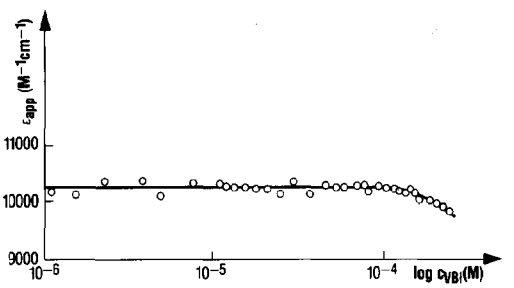

Fig. 1. Concentration dependence of apparent absorption coefficient ε_{app}.

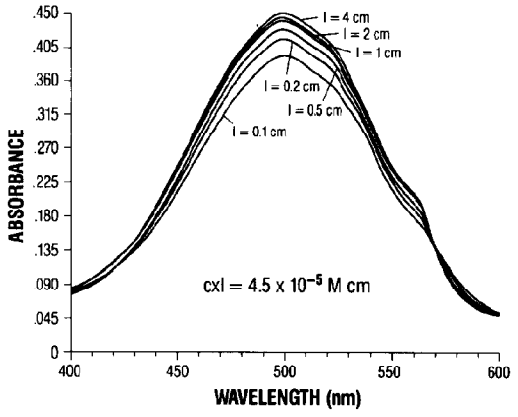

Fig. 2. Absorption spectra of VBI solutions at constant $c \times l$.

value of 4.5×10^{-5} M cm. An isosbestic point at $\lambda = 573$ nm, with the appearance of a new absorption band at $\lambda \simeq 585$ nm is characteristic to the dimer formation. The data obtained from Fig. 2 are given in Table 1.

The evaluation of the self-association of anthracycline ligands is a prerequisite for the study of their interaction with a polymer. The literature describes several computation methods for estimating the equilibrium constant of dimerization from the experimental absorption values under the condition constant $c \times l$ [4–7] applied here.

First we calculated the dimerization constant K_d using the method described by Tipping et al. [6] starting from the following equations:

$$K_d = c_d / c_m^2, \tag{1}$$

$$A = l c_t \varepsilon_{app} = l(\varepsilon_m c_m + 2\varepsilon_d c_d), \tag{2}$$

Table 1

Experimental absorption data at $\lambda = 500$ nm and $c \times l = 4.5 \times 10^{-5}$ M cm

l (cm)	c_t (M)	A_{500}	ε_{app}^{500} (M^{-1} cm^{-1})
0.1	4.5×10^{-4}	0.3934	8742.22
0.2	2.25×10^{-4}	0.4155	9233.33
0.5	0.90×10^{-4}	0.4275	9500.00
1.0	0.45×10^{-4}	0.4393	9762.22
2.0	0.225×10^{-4}	0.4438	9862.22
4.0	0.1125×10^{-4}	0.4497	9993.33

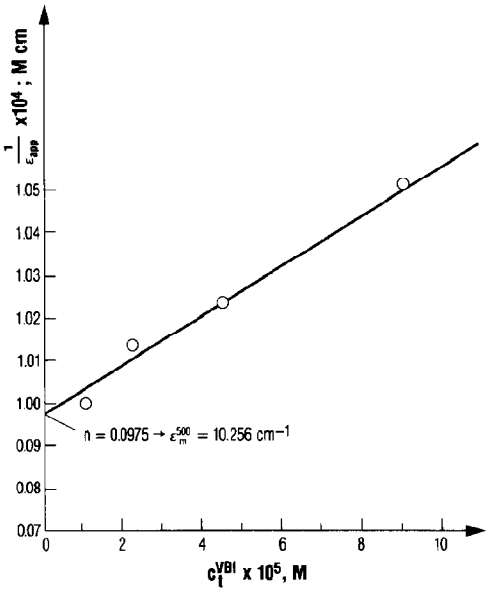

Fig. 3. The plot $1/\varepsilon_{app}$ vs. total concentrations of VBI.

where c_t, the total concentration of VBI in terms of monomer, is:

$$c_t = c_m + 2c_d. \tag{3}$$

In eq. (2) $2\varepsilon_d$ is the molar absorption coefficient of the dimer. The symbols ε_m, ε_d, ε_n in the literature usually represent the extinction coefficient per monomeric unit in an associate.

Extrapolation of a plot $1/\varepsilon_{app}$ against c_t to $c_t \to 0$ gives $1/\varepsilon_m$ as the intercept of the ordinate, as Fig. 3 shows, with a value of 10256 M^{-1} cm^{-1} for ε_m in good agreement with those obtained from the spectrophotometric titrations.

Elimination of c_m using (1) and (3), respectively (2) and (3), followed by elimination of c_d yields the expression:

$$\left(\frac{c_t}{\varepsilon_m - \varepsilon_{app}}\right)^{1/2} = \frac{1}{\varepsilon_m - \varepsilon_d}\left[c_t(\varepsilon_m - \varepsilon_{app})\right]^{1/2} + \left[\frac{1}{2K_d(\varepsilon_m - \varepsilon_d)}\right]^{1/2} \tag{4}$$

Thus for dimerization, a plot of $(c_t/(\varepsilon_m - \varepsilon_{app}))^{1/2}$ against $(c_t(\varepsilon_m - \varepsilon_{app}))^{1/2}$ should give a straight line, the intercept n and slope α of which

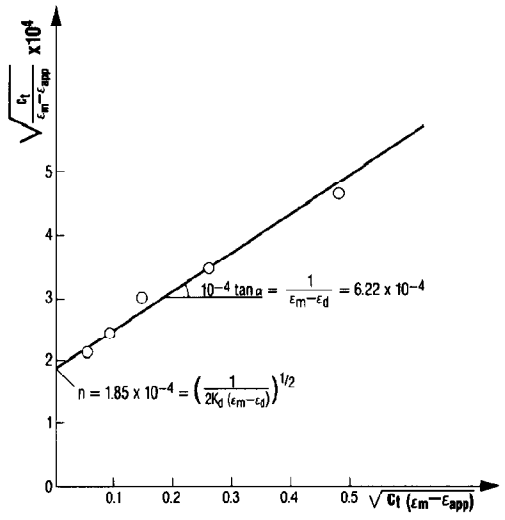

Fig. 4. Tipping's plot of eq. (4).

can be used to calculated K_d and ε_d. Our results at $\lambda = 500$ nm give a linear fit to eq. (4), $R = 0.995$, when one does not include the highest concentration 4.5×10^{-4} M where the equilibrium is probably too much shifted towards the dimer and perhaps other aggregates.

The experimental data yield an equilibrium constant for dimerization $K_d = 9087$ M^{-1} and a value of 17036 M^{-1} cm^{-1} for the molar absorption coefficient of the dimer, that is $\varepsilon_d = 8518$ M^{-1} cm^{-1} if ε refers to one mole of monomeric units.

We have considered our data by using for the estimation of the above parameters the method proposed by Schwarz et al. [4] which starts from the same simple dimerization model. The equation they used is a rearrangement of the previous equation (4):

$$\left(\frac{\varepsilon_m - \varepsilon_{app}}{c_t}\right)^{1/2} = \left(\frac{2K_d}{\Delta\varepsilon}\right)^{1/2} \left[\Delta\varepsilon - (\varepsilon_m - \varepsilon_{app})\right] \tag{5}$$

which can be written as follows to yield a different linear expression between the measurable quantities:

$$\left(\frac{\varepsilon_m - \varepsilon_{app}}{c_t}\right)^{1/2} = (2K_d\Delta\varepsilon)^{1/2} - \left(\frac{2K_d}{\Delta\varepsilon}\right)^{1/2} \times (\varepsilon_m - \varepsilon_{app}), \tag{6}$$

where $\Delta\varepsilon = \varepsilon_m - \varepsilon_d$ (any ε refers to one mole of monomeric units).

A plot of $((\varepsilon_m - \varepsilon_{app})/c_t)^{1/2}$ against $(\varepsilon_m - \varepsilon_{app})$ yields an intercept n and a slope tan α, from which $\Delta\varepsilon$ and therefore ε_d as well as K_d may be calculated as Fig. 5 shows.

One observes that $\Delta\varepsilon = -n/\tan\alpha = 1611$ M^{-1} cm^{-1} so that one calculates $\varepsilon_d = 8693$ M^{-1} cm^{-1} which is comparable with that obtained from Tipping's plot (8518 M^{-1} cm^{-1}). Our computation gave a dimerization constant $K_d = 9624$ M^{-1} a little higher than that from Tipping's equation (9087 M^{-1}). We consider an average value of 9355 M^{-1} for the dimerization constant K_d.

Chaires et al. [8] investigated the self-association of daunomycin, also using spectrophotometric measurements to determine the dimerization constant K_d. They affirm that this method does

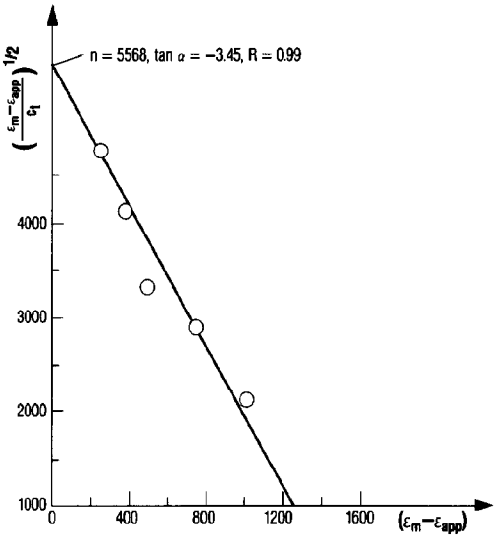

Fig. 5. The plot $((\varepsilon_m - \varepsilon_{app})/c_t)^{1/2}$ vs. $(\varepsilon_m - \varepsilon_{app})$ of Schwarz's equation.

not provide a critical test of the dimerization model because aggregation beyond a dimer can also account for the observed changes in absorbance. Independent evidence is thus required for a definitive assignment of the association model. Sedimentation equilibrium experiments using an indefinite association model, supported also by NMR measurements, gave only a half of the K_d value from spectrophotometric data. Therefore they concluded that in their system higher aggregation must occur.

One year later, Walter et al. [7] studied the self-association of iremycin, from absorption measurements only. They started from a monomer–dimer equilibrium because within the concentration range used, matrix-rank analysis [9] of the iremycin spectra did not suggest a rank higher than two. The parameters of dimerization were obtained simultaneously at three characteristic wavelengths from the measured absorbance by a curve-fitting program.

We tried to apply the method of Zadorojnaia et al. [5] which permits to determine the degree of aggregation n, from absorption measurements by using the equation

$$\log \beta c = \log nK_n + n \log(1-\beta)c, \qquad (7)$$

β being the degree of aggregate formation and K_n the equilibrium constant of aggregation. From the linear plot of log βc against $\log(1-\beta)c$ we obtained always from the slope a value of $n \simeq 2$, but K_d varied unsatisfactorily within an order of magnitude $10^3 - 10^4$ M^{-1}. Another support for the simple monomer–dimer equilibrium model that we adopted in this study is offered by the values of dimerization constant K_d calculated simultaneously at three different wavelengths.

Table 2

Parameters of violamycin dimerization

λ (nm)	ε_m (M^{-1} cm^{-1})	ε_d (M^{-1} cm^{-1})	K_d (M^{-1})
500	10878	9564	9256
540	7057	6449	9466
563	4634	3605	9300
			$\bar{K}_d = 9341$

Starting from eqs. (1), (2) and (3) calculated by a curve fitting program (as described before) K_d, ε_m and ε_d and obtained from the spectrophotometric titration the data listed in Table 2.

One observes that in the limit of the experimental errors K_d does not change with the wavelength which pleads for the presence of a single species of aggregate, viz. the dimer. The average value of 9341 M^{-1} is in good agreement with those from our experiments carried out at constant $c \times l$, namely 9355 M^{-1}.

3.3. Temperature-jump measurements

Kinetic measurements for the dimerization process were done with the T-jump method. The relaxation curves of three samples of 1×10^{-5} M, 4.2×10^{-5} M and 7.3×10^{-5} M, prepared in the same buffer ($I = 0.2$ M), were measured at $\lambda =$ 500 nm. Unfortunately, the amplitudes were small so that the determination error of the rate constants k_{12} and k_{21}, and therefore of the dimerization constant K_d, were somewhat higher than those obtained from spectrophotometrical measurements. One remarks also that the absorbance of the sample varied after 2–5 temperature jumps under irradiation, violamycin being photosensitive.

Table 3 points out our experimental data characteristic for the equilibrium

$$2M \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} D \qquad (8)$$

by using eq. (9) and plotting τ^{-2} against c_t^{VBI}:

$$\tau^{-2} = 8k_{12}k_{21}c_t + k_{21}^2 \qquad (9)$$

Figure 6 represents the plot of these data. The intercept is $k_{21}^2 = 0.34 \times 10^8$ s^{-2} which leads to $k_{21} = 0.583 \times 10^4$ s^{-1} and the slope is $8k_{12}k_{21} = 2.32 \times 10^{12}$ M^{-1} s^{-2}, or $k_{12} = 4.974 \times 10^7$ M^{-1} s^{-1}. If we rewrite eq. (9), taking into account the condition of mass conservation and the kinetic definition of K_d, we get

$$\tau^{-2} = k_{21}^2(8K_d c_t + 1) \qquad (10)$$

One observes that the point of intersection with the abscissa gives a value of $-(8K_d)^{-1}$.

Table 3

T-jump experimental data, $\lambda = 500$ nm, $T = 27$°C

c_t, M	1.0×10^{-5}	4.2×10^{-5}	7.3×10^{-5}
τ^{-1}, s^{-1}	0.803×10^4	1.08×10^4	1.45×10^4
τ^{-2}, s^{-2}	0.645×10^8	1.166×10^8	2.103×10^8

This permits the direct evaluation of $K_d = 8550$ M^{-1}, similar with the value obtained from the ratio $k_{12}/k_{21} = 8532$ M^{-1}. This value is somewhat lower than that obtained from the spectrophotometrical measurements at 20.5°C (9355 M^{-1}), because the dimer dissociates with increasing temperature. These measurements at two different temperatures, although obtained with different methods, allow an estimate of the standard reaction enthalpy of the dimerization $\Delta H^0 = -6.4$ kJ mol^{-1}, indicating that the largest contribution to the value of $\Delta G^0 = -22.5$ kJ mol^{-1} at 20.5°C is of entropic nature, corresponding to a positive entropy increase of $\Delta S^0 = 54.5$ J mol^{-1} K^{-1} upon dimerization. The fact that violamycin at neutral pH carries two positively charged aminoglycosides compared to only one in the case of other anthracycline antibiotics may be lowering the binding enthalpy and influencing the solvation structure of the dimer. Chaires et al. [8] report $\Delta H^0 = -33.4$ kJ mol^{-1} and $\Delta S^0 = -59.7$ J mol^{-1} K^{-1} for their association model of daunomycin. This model allows aggregation beyond the dimer, indicated by the sedimentation equilibrium experiments. The most probable structure of the aggregate is then a helical arrangement of the aminoglycosides on the periphery of stacked anthracycline rings. In the case of VBI such a structure beyond the dimer would be inhibited by the excessive steric hindrance induced by the two aminoglycoside substituents. The origin of the more positive reaction entropy (by almost 14 R) is not clear, although it must be understood that this is a sum of several contributions, including release of solvent molecules solvating the monomers and changes in the surrounding solvent configuration and in the counterion distribution.

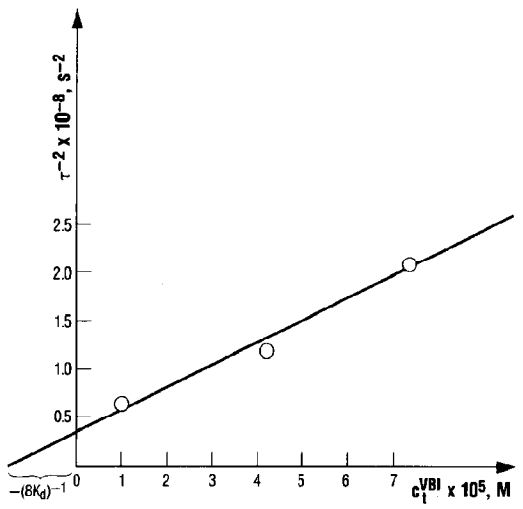

Fig. 6. The plot of eq. (9).

The absence of a spectral shift does not exclude a structure in which the chromophores are stacked in an antiparallel fashion with their transition moments perpendicular to the stacking axis. The appearance of a new optical transition in the dimer is clearly indicated by the isosbestic point in the absorption data. The lowering of the extinction coefficient of the main transition also shows a coupling between the two chromophores. Similar spectral behaviour is observed for the association of daunomycin. For this compound the changes in the NMR chemical shifts of aromatic protons, which can be attributed to aromatic ring current shifts, favor the stacking model [8].

The VBI parameters obtained from our spectrophotometrical measurements are compared in Table 4 with the data reported in the literature for other anthracycline antibiotics [9].

The data for violamycin fit well into this table although the authors mentioned used another buffer, namely McIlvaine (pH 6; 0.16 M, Na^+).

Table 4

Dimerization parameters determined at the absorption maximum of the monomer [7]

Parameter	Iremycin	Daunomycin	Adriamycin	Violamycin
ε_m, M^{-1} cm^{-1}	13346	10781	12682	10250
ε_d, M^{-1} cm^{-1}	6842	7497	7962	8639
K_d, M^{-1}	1838	6145	11093	9350

The dimerization constant K_d of violamycin has an intermediate value between daunomycin and adriamycin, the last one forms the most stable dimers. These K_d equilibrium constants have to be taken into account in the evaluation of the interaction of the antibiotics with DNA and other biological macromolecules and cell structures.

Acknowledgement

We thank Dr. D.G. Strauss for the kindness with which he offered us the necessary violamycin to undertake this study.

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