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Thermodynamic Analysis of Self-association of Actinocin Bis(2-dimethylaminoethyl)amide in Aqueous Solution by ^{1}H NMR Spectroscopy

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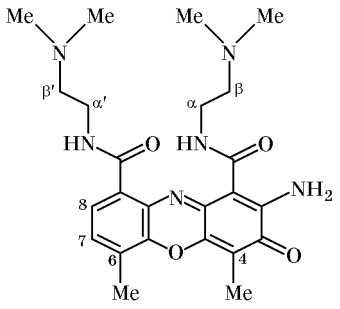
Abstract—Self-association of a synthetic antibiotic, actinocin bis(2-dimethylaminoethyl)amide (ActII), in aqueous solution was studied by one- and two-dimensional ^{1}H NMR spectroscopy (500 MHz).

The design of new antitumor agents requires that the mechanisms underlying their biological activity be understood on the molecular and cellular level. The results of studies in the field of synthetic phenoxazine antibiotics showed that their antitumor activity strongly depends on the structure of side chains attached to the phenoxazine chromophore [1]. Our studies by flow cytofluorimetry of induced apoptosis and cellular cycle phases in MOLT-3 human leukemia cells indicated that the antitumor activity of the synthetic antibiotic actinocin bis(2-dimethylaminoethyl)-amide (ActII) having an aminoalkyl side chain with two methylene units is considerably higher than the activity of its analog ActIII having three methylene groups in the side chain [1, 2]. ActIII exhibits an appreciable cytotoxic effect in the concentration range from 1 to 9 mM [1]. According to the results of studies of interactions between ActIII and DNA in aqueous salt solution by electron absorption spectroscopy in the ultraviolet and visible regions, the most probable mechanism of complex formation is intercalation of the phenoxazine chromophore (ligand) between the planes of nitrogen bases [1, 3]. Structural similarity of ActII and ActIII allows us to presume that ActII is also bound by DNA via intercalation. Quantitative analysis of specific features intrinsic to complex formation between synthetic antibiotics and DNA requires that parameters of self-association of the aromatic molecules be known [4–6]. These data are also important for estimation of the role of stacking interactions between aromatic ligands and DNA bases in the intercalated complex [7].

In the present work we examined self-association of ActII in aqueous solution by one- (1D) and two-dimensional (2D) ^{1}H NMR spectroscopy (500 MHz). The structural and thermodynamic parameters for aggregation of the phenoxazine antibiotic were analyzed in a way similar to our previous studies on aromatic dyes and antibiotics [2, 8, 9], i.e., using concentration and temperature dependences of proton chemical shifts.



ActII

The proton signals of ActII were assigned by analysis of the 2D-TOCSY and 2D-ROESY spectra [4–6]. In the 2D-ROESY spectrum (Fig. 1) we observed two groups of cross peaks. The first of these includes cross peaks arising from 8-H–6-CH_3, 4-CH_3–α-CH_2, 4-CH_3–β-CH_2, and 6-CH_3–β′-CH_2; the second group consists of 8-H–4-CH_3, 7-H–β-CH_2, and β-CH_2–6-CH_3 (denoted with arrows in Fig. 1). The latter should be attributed to intermolecular interactions between closely located protons in ActII aggregates, provided that the NMR data were acquired

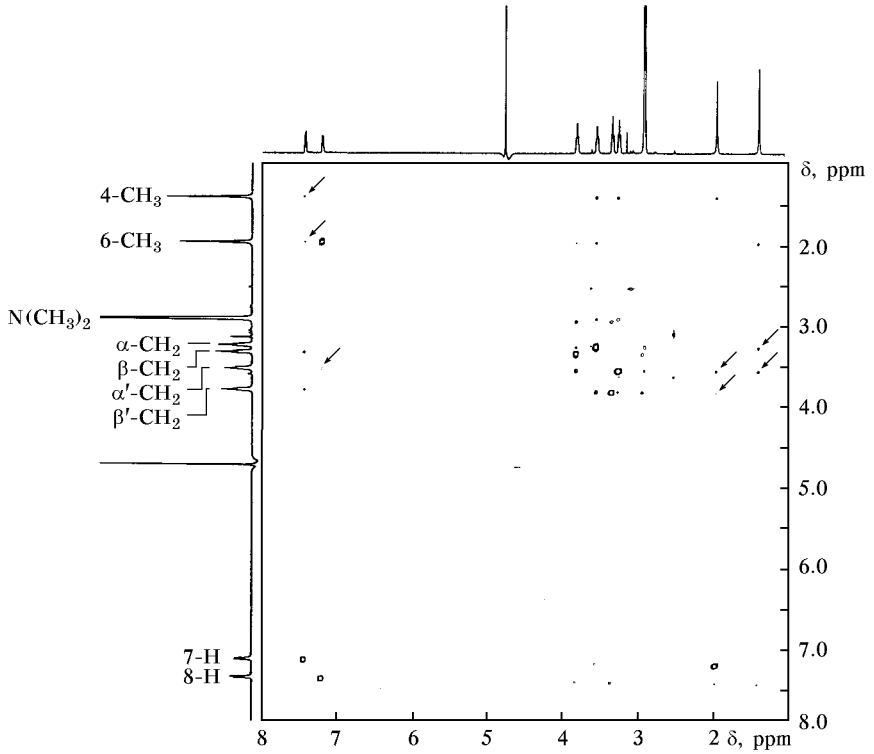


Fig. 1. 2D–ROE spectrum (500 MHz, τ_m 240 ms) of a solution of ActII (x_0 = 2.0 mM, T = 298 K). Intermolecular cross peaks are denoted with arrows.

at a small mixing period which eliminates spin diffusion effects. The first-group cross peaks suggest the presence in solution of self-associates with antiparallel orientation of the ActII chromophores, while the second group of couplings indicates formation of structures which are also characterized by antiparallel chromophore orientation, but the chromophores therein are additionally turned through an angle of 180° about their transverse axis, as was observed for ActIII aggregates [2]. It should be noted that the two kinds of intermolecular complexes formed by stacking interaction between the chromophores were also found in the association of daunomicin and phenanthridine dye ethidium bromide in aqueous solution [10].

Figure 2 shows the experimental concentration dependences of proton chemical shifts of ActII. These data also indicate association of ActII via stacking interaction of the chromophores which are oriented antiparallel with respect to each other. In fact, the resonance signals belonging to protons of the ActII chromophore shift upfield as the concentration rises. Such a pattern is typical of stacking association of aromatic molecules in solution [8]. Here, the 4-CH_3 protons are shielded to an appreciably greater extent than the 6-CH_3 protons. This means that the 4-methyl group in the associate is located in the area affected by electromagnetic field of the benzene ring.

As previously [8, 9], the experimental data were interpreted in terms of the infinite-dimensional noncooperative self-association model, according to which the equilibrium constants K_j for reaction (1) are assumed to be equal for any j: $K_1 = K_2 = ... = K_j = K$.

$$X_j + X \overset{K_j}{\longleftrightarrow} X_{j+1} \qquad (1)$$

Then, the dependence of chemical shift δ on the initial concentration x_0 is given by Eq. (2) [8]:

$$\delta = \delta_m + (\delta_i - \delta_m)\left[\frac{2K x_0 + 1 - \sqrt{4K x_0 + 1}}{2K x_0}\right], \qquad (2)$$

where δ_i is the proton chemical shift for the antibiotic molecule in the aggregate, and δ_m is the same for the monomeric molecule, i.e., at infinite dilution. The experimental data were treated by the variational procedure [8, 11]. The parameters δ_m, δ_i, and K in Eq. (2) were determined by minimization of the quadratic residue function [11]. The numerical proce-

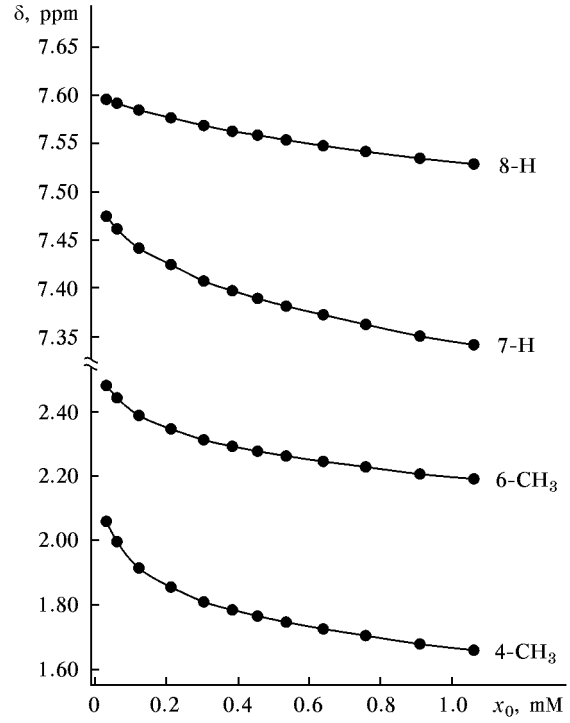


Fig. 2. Experimental concentration dependences of proton chemical shifts in the spectrum of ActII at 298 K.

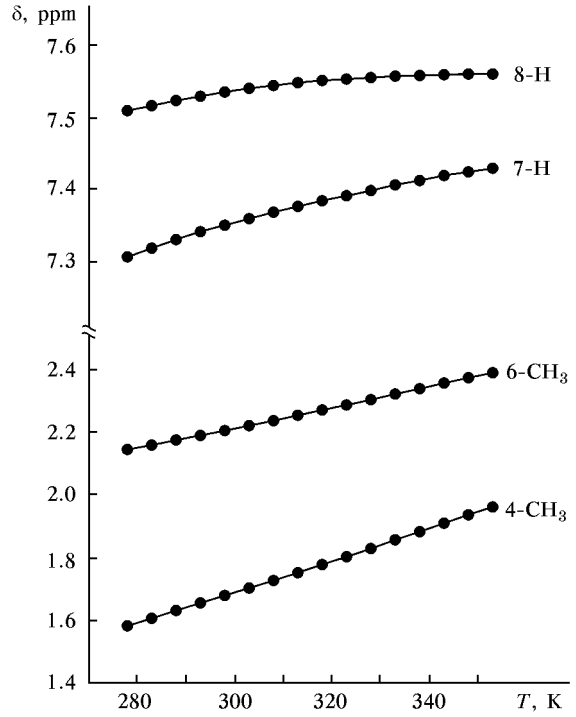


Fig. 3. Experimental temperature dependences of proton chemical shifts in the spectrum of ActII at x_0 = 0.91 mM.

dure for minimization of the residue function was described in [8, 11]. The calculated parameters for unexchangeable protons in the ActII chromophore are given in table.

The equilibrium constants K for self-association of ActII in 0.1 M phosphate buffer, pD 7.1, is somewhat smaller than the corresponding value for ActIII, determined under similar conditions [2]. This may be due to increased electrostatic repulsion between the side chains in ActII, which are shorter than those in ActIII and are positively charged in solution.

In order to estimate the probability for formation of ActII aggregates of a higher order than dimers, the experimental data were treated in terms of the infinite-dimensional cooperative self-association model where the equilibrium constants of reaction (1) are assumed to be equal for all $j \geq 2$ ($K_2 = K_3 = ... = K_j = K$) and $K_1 = \sigma K$ [8]. When $\sigma = 1$, we have the noncooperative model considered above. A system is cooperative at $\sigma < 1$, if formation of dimers creates energetically favorable conditions for the subsequent association of molecules, and it is anticooperative at $\sigma > 1$. Such model gives the following concentration dependence of chemical shifts δ [Eq. (3)]:

$$\frac{\delta - \delta_m}{\delta_i - \delta_m} = 1 - \frac{x_1}{x_0} - \frac{\sigma K x_1^2}{x_0(1 - K x_1)}. \qquad (3)$$

Here, x_0 is the initial molar concentration, and x_1 is the molar concentration of monomeric species in solution. Equation (3) contains four unknown parameters: δ_m, δ_i, σ, and K, which should be determined from the experimental concentration dependences of chemical shifts δ. The corresponding procedure was described in detail in [8, 11]. The values calculated according to the cooperative model, are also given in table. It is seen than the values of σ for ActII and ActIII [2] coincide within the error in their determination, which may be exlained by structural similarity of their molecules. It should be noted that the cooperativity parameter for self-association of actinomycin D D [12] containing bulky pentapeptide rings as side chains (σ 1.5) strongly differs from the σ values found for synthetic phenoxazine antibiotics having aminoalkyl side chains. On the basis of the data given in table we can also conclude that the induced proton chemical shifts $\Delta\delta = \delta_m - \delta_d$ for ActII are fairly similar to those calculated for ActIII [2], which suggests some similarity in the steric structures of ActII and ActIII dimers in aqueous solution.

The thermodynamic parameters for self-association of ActII were determined from the experimental temperature dependences of chemical shifts δ_i of unexchangeable protons (Fig. 3). The temperature dependence of δ_i is described by Eq. (4) [8].

$$\delta_i(T_j) = f_m(T_j)\delta_{mi} + f_a(T_j)\delta_{ai}. \quad (4)$$

Here, δ_{mi}, δ_{ai} and $f_m(T_j)$, $f_a(T_j)$ are proton chemical shifts and equilibrium mole fractions of monomeric species of the antibiotic and its associates, respectively, at a temperature T_j. Equation (4) assumes that δ_{mi} and δ_{ai} for protons of the phenoxazine chromophore do not depend on temperature in the examined temperature range. An analogous pattern was observed by us previously [8, 9] for aromatic molecules of dyes and antibiotics in aqueous solution.

In Eq. (4), the effect of temperature on δ values is taken into account through mole fractions f_m and f_a which are unambiguously related to the equilibrium association constant K and are functions of temperature. From the temperature dependence of the equilibrium constant we can find the corresponding thermodynamic parameters, enthalpy ΔH and entropy ΔS for self-association of ActII molecules. The procedure for calculation of ΔH and ΔS was described in detail in [8]. The averaged thermodynamic parameters for self-association of ActII (see table) indicate that this process is exothermic. It is believed that self-association of aromatic molecules is governed mainly by dispersion interactions between their chromophores, which are characterized by negative values of both enthalpy and entropy [13–15]. The absolute values of the enthalpies of self-association of ActII (see table) and ActIII [2] coincide within the error in their determination. Moreover, the enthalpy of self-association of actinomycin D [12] also coincides with ΔH found for the synthetic phenoxazine antibiotics. Therefore, we conclude that the main contribution to the enthalpy of association of their molecules is provided by stacking interactions of phenoxazine chromophores. On the other hand, self-association of ActII is characterized by a smaller absolute value of ΔS, as compared to ActIII. This may be due to both differences in electrostatic repulsion between antibiotic molecules in the aggregates and somehat weaker structurization of water around the dimeric complex, caused by hydrophobic interactions (the side chain in ActII is shorter than in ActIII by one CH_2 group). Thus a conclusion can be drawn that variation of the length of the side chain in phenoxazine antibiotics exerts a certain effect on electrostatic and hydrophobic interactions which accompany association of their molecules.

Calculated parameters of self-association of actinocin bis(2-dimethylaminoethyl)amide in 0.1 M phosphate buffer (D_2O, pD 7.1)

Infinite-dimensional noncooperative model				Infinite-dimensional cooperative model			
proton	δ_m, ppm	δ_i, ppm	K, 10^3 l/mol	δ_m, ppm	δ_i, ppm	K, 10^3 l/mol	σ
			T = 298 K				
8-H	7.61	7.35	2.8 ± 1.1	7.60	7.32	2.5 ± 1.6	1.25 ± 0.01
7-H	7.49	7.19		7.49	7.15		
6-CH_3	2.53	1.98		2.53	1.94		
4-CH_3	2.12	1.37		2.16	1.39		
			T = 308 K				
8-H	7.60	7.32	2.1 ± 0.9	7.60	7.29	2.0 ± 1.4	1.2 ± 0.05
7-H	7.49	7.21		7.49	7.17		
6-CH_3	2.55	2.01		2.55	1.98		
4-CH_3	2.16	1.40		2.20	1.43		

Thermodynamic parameters

$-\Delta G^0$, kJ/mol	$-\Delta H^0$, kJ/mol	$-\Delta S^0$, J mol^{-1} K^{-1}
19.7 ± 1.0	29.7 ± 3.6	32.4 ± 9.5

EXPERIMENTAL

The 1D and 2D ^{1}H NMR spectra were measured on a Bruker DRX spectrometer operating at 500 MHz. The residual HOD signal was saturated during the acquisition period. The chemical shifts were measured relative to DSS (2,2-dimethyl-2-silapentane-5-sulfonic acid), and tetramethylammonium bromide was used as internal reference. The concentration dependences of proton chemical shifts were determined at 298 and 308 K in the range of ActII concentrations from 0.909 to 0.030 mM; the temperature dependences of proton chemical shifts were determined in the temperature range from 278 to 353 K. The procedures for sample preparation and one- and two-dimensional (TOCSY, ROESY) ^{1}H NMR experiments were described in detail in [4, 5].

Actinocin bis(2-dimethylaminoethyl)amide was synthesized according to the procedure reported in [16, 17], by catalytic hydrogenation of the corresponding 3-benzyloxy-4-methyl-2-nitrobenzamide and subsequent oxidation of the resulting *o*-aminophenol derivative with *p*-benzoquinone. The structure of the product was confirmed by elemental analysis and IR, electron absorption (visible region) [16, 17], and 1D and 2D ^{1}H NMR spectra. The antibiotic was lyophilized from D_2O (isotope purity 99.95%, from Sigma) and was dissolved in 0.1 M deuterated phosphate buffer (pD 7.1, 0.1 M NaCl) containing 10^{-4} mol/l of ethylenediaminetetraacetic acid.

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