The Average Unwinding Angle of DNA Duplex Produced by the Binding of Chromomycin A_3

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The effect of chromomycin A_3 binding on the geometry of DNA duplex (plasmid pBR322) has been examined using topoisomerase I relaxation followed by gel electrophoresis. To determine the equilibrium constant of this drug–DNA binding–dissociation reaction in the same concentration range (*ca.* 10^{-5} M) in the same buffer as those for the topoisomerase reaction (at 37 °C), fluorescence measurements were made of the same plasmid–drug system, followed by a Scatchard plot and an analysis using McGhee–von Hippel's exclusion site model. The binding constant has been found to be 3.8×10^5 M^{-1} in the particular buffer (buffer-T) at 37 °C, and the number of base pairs involved in the site of one chromomycin molecule on the duplex has been found to be 5. It has been concluded that one chromomycin molecule, bound to the duplex, unwinds it by 11.8±1.1 degrees. In addition, the enthalpy of binding was determined to be 31.81 kJ/mole using a titration calorimeter with a more concentrated (6.2 mM) solution.

Key words unwinding of DNA duplex; chromomycin A_3; plasmid; topoisomer; calorimetry

This is the second report of our series of studies,[1] attempting a precise determination of the unwinding angle in the DNA duplex caused by every type of ligand binding. Unwinding angle is considered to be an essential parameter, useful in constructing a mechanistic idea of the molecular biology of a DNA–ligand interaction.

Chromomycin A_3 is one of the antibiotics produced by *Streptomyces griseus* and is known to have antitumor activity.[2] Miyamoto *et al.*[3] elucidated its chemical structure (Fig. 1), and a number of biochemical studies have been reported on it.[4] An important insight into the structure of the chromomycin–DNA complex emerged from a two-dimensional NMR study of chromomycin complex with self-complementary $d(T_2G_2C_2A_2)_2$ duplex by Gao and Patel.[5] They showed that a Mg^{2+} coordinated chromomycin dimer is bound at the minor groove of the DNA oligomer duplex, and the total unwinding angle caused by this binding is 23°. This angle, however, is likely to be assigned only to this particular base-sequence, TTGGCCAA, and also it is likely to involve an end-effect, because both ends of this sequence are free in their sample DNA. In our present study, we have chosen plasmid pBR322, a closed circular duplex with 4362 base pairs found in *Escherichia coli*, which is a more general and natural DNA, as the subject of our unwinding study.

Experimental

Materials The sample of plasmid pBR322 was prepared as described previously.[1] pFP332 (2701 bp) used for isothermal titration calorimetry (ITC) was prepared also using a QIAGEN Plasmid Mega Kit (QIAGEN GmbH, Germany). Topoisomerase I from calf thymus was purchased from Takara Shuzo Co., and used without further purification. Chromomycin A_3 was supplied by Sigma Chemical Co.

The chromomycin plus plasmid reactions were produced in a buffer, which is appropriate for topoisomerase I activity measurement. This buffer

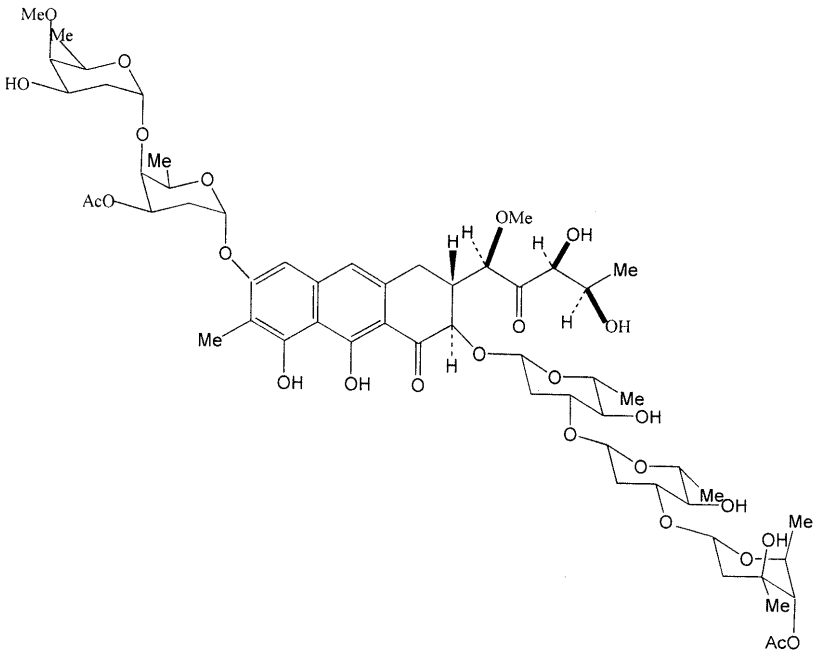

Fig. 1. Structure of Chromomycin A_3

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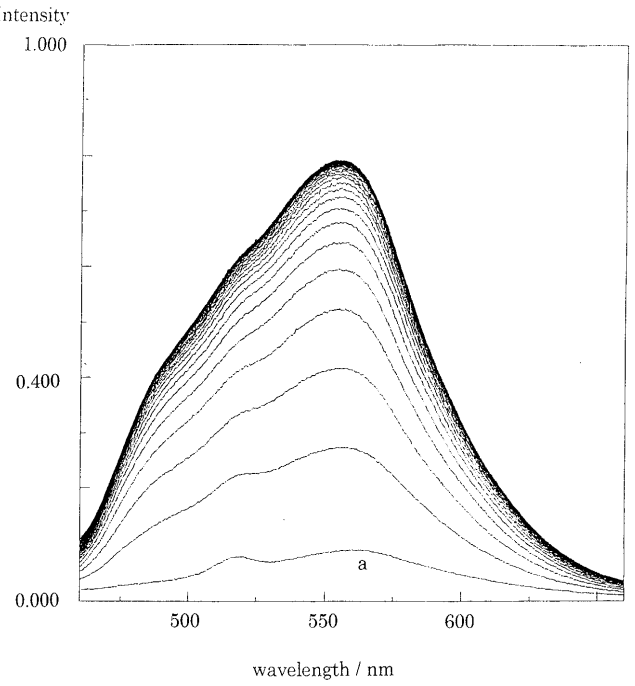

Fig. 2. Fluorescence Spectra of Chromomycin A_3 in Buffer-T at 37 °C, Excited at 440 nm

a, chromomycin A_3 1.34×10^{-6} M only. (Curves with no labels): solutions consist of 3000 μl of 1.34×10^{-6} M chromomycin+5 μl$\times J$ of 2.09×10^{-3} M (in base pairs) of pBR322 DNA, where J=1—40.

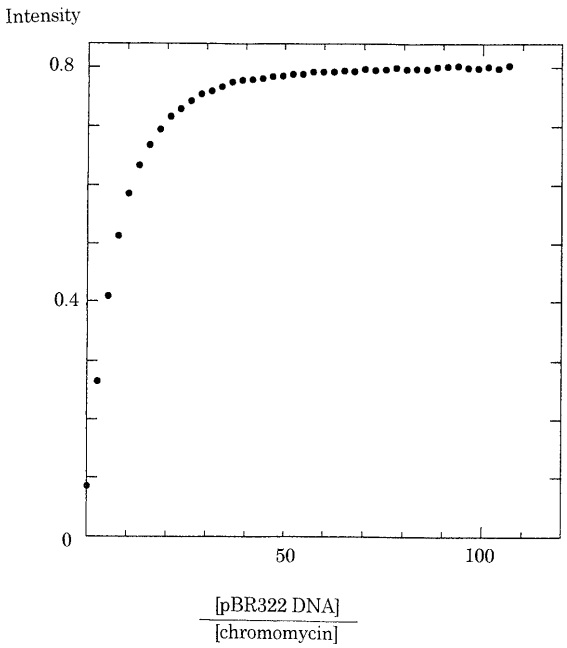

Fig. 3. Fluorescence Intensity at 560 nm of Chromomycin A_3 1.34×10^{-6} M in Buffer-T at 37 °C Plotted against the Amount of pBR322 DNA Added

The amount of DNA here is given by the ratio of concentration of DNA base pair/concentration of chromomycin A_3.

contains 35 mM Tris–HCl (pH=8.0), 72 mM KCl, 5 mM $MgCl_2$, 5 mM dithiothreitol (DTT), 5 mM spermidine, and 0.01% bovine serum albumin (BSA) (hereafter called buffer-T).

Fluorescence Measurements A Jasco FP-770 spectrometer was used. Chromomycin was dissolved in buffer-T, 3000 μl of this solution was then placed in a cuvette, and this was mounted on the fluorescence spectrophotometer. The fluorescence was excited at 440 nm, and the emission spectrum was observed in the 460—660 nm region. A particular amount of DNA solution was added to the solution, and the spectroscopic measurement was made after stirring. The temperature of the solution was kept at 37 °C using a SCINICS Cool Circulator CH-201.

Electrophoresis Analysis Three stock solutions of chromomycin A_3, with concentrations of 1.399×10^{-5}, 2.639×10^{-5}, and 4.050×10^{-5} M, were prepared by the use of sterilized, distilled water. The concentration was determined by the measurement of the absorption spectrum, on the assumption that the molar extinction coefficient at 406 nm, ε_{406}, of chromomycin is 10000.[6]

The samples were prepared as follows. To 35 μl of sterilized, distilled water, 5 μl of 10-times concentrated buffer-T (without BSA), 5 μl of 0.1% BSA, 0.1 μl of pBR322 (0.5 μg/μl), and 2 μl of chromomycin A_3 solution were added in this sequence. The solution mixture was incubated at 37 °C for 10 min to complete the binding reaction. Next, 2 μl of 6 units/μl topoisomerase I was added to this solution, and this was incubated at 37 °C for 2 h, to cause a complete relaxation of the supercoiled plasmid (with drug bound). Then, the drug and enzyme were removed through phenol extraction, and DNA was isolated by ethanol precipitation. The isolated DNA was then subjected to 1% agarose gel electrophoresis. The buffer used here was TBE (90 mM Tris–HCl and 2 mM EDTA). The experiment was done by imposing 40 V, at 22 °C and for 20 h. After that, the gel was stained with ethidium bromide and subjected to photography.

The mobility of pBR322 DNA is related to its writhing number τ. The relation was examined in detail using agarose gel containing various amounts of chloroquine. When 0.5 μg/ml of chloroquine, for example, was added in the agarose gel as well as in the TBE buffer, the τ value of pBR322 was found to be elevated by 10. It was found that pBR322 DNA with $\tau=+1$, $+2$, ... had a slightly greater mobility than pBR322 DNA with $\tau=-1$, -2, ..., respectively.

ITC An MCS isothermal titration calorimeter from MicroCal, Inc. was used. To a 0.5 mg/ml solution of pFP332 DNA, a 6.20 mM solution of chromomycin A_3 was injected 20 times at 37 °C with 9.003 μl each time with an injection duration of 11.23 seconds and the time between injections 600 seconds. Here, the solvent consisted of 35 mM Tris–HCl, pH=8.0, 72 mM KCl, 5 mM $MgCl_2$, and 9% methanol. Methanol was necessary to obtain the concentration of chromomycin as high as 6.20 mM.

Results

Figure 2 shows a result of the fluorescence spectral measurements. As seen here, 2.09×10^{-6} M chromomycin A_3 gives only weak fluorescence (curve a, Fig. 2), but on adding pBR322 DNA its fluorescence becomes stronger. The fluorescence intensity at 560 nm is plotted against the amount of DNA added in Fig. 3. After the DNA/chromomycin concentration ratio reaches 40, the fluorescence intensity shows almost no increase any more. Here, almost all the chromomycin molecules are considered to be bound by DNA, and this fluorescence is considered to represent that of the chromomycin bound to DNA. Our present aim is to determine the concentrations of the free drug (L_f) and drug bound to DNA (L_b) in the solution with DNA/drug concentration ratio 0—20. The fluorescence spectra for this range of the concentration ratio are shown in Fig. 4. From this result the binding ratio γ was calculated and γ/L_f was plotted against γ (Scatchard plot[7]). The result is shown in Fig. 5. For the analysis of this plot, we adopted the exclusion site model of McGhee and von Hippel,[8] who proposed the following equation,

$$\frac{\gamma}{L_f}=K(1-n\gamma)\left[\frac{1-n\gamma}{1-(n-1)\gamma}\right]^{n-1} \quad (1)$$

where n is the number of base pairs occupied by one drug molecule bound to DNA duplex, and

$$K=\frac{[\text{bound drug}]}{[\text{free drug}]\,[\text{free site}]} \quad (2)$$

is the equilibrium constant. As can be seen in Fig. 5, a McGhee–von Hippel curve (Eq. 1) fitted the experimental

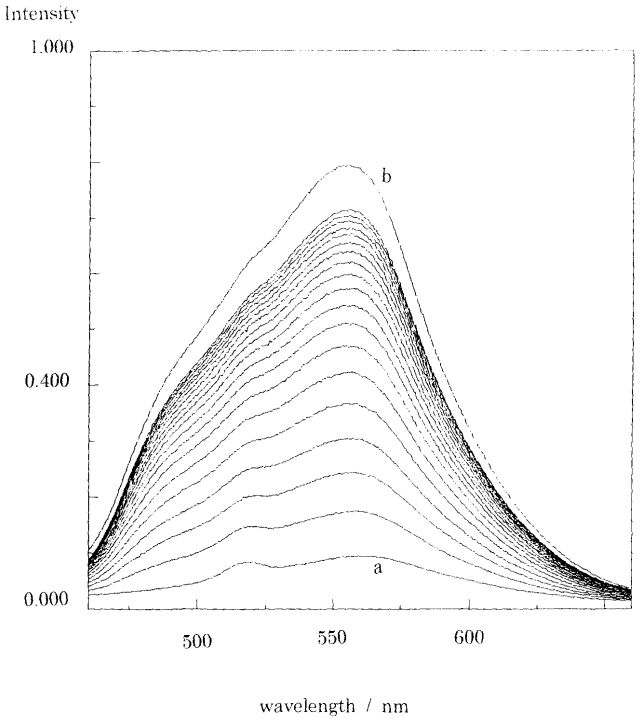

Fig. 4. Fluorescent Spectra of Chromomycin A_3 in Buffer-T at 37 °C, Excited at 440 nm

a, chromomycin A_3 1.34×10^{-6} M only. (Curves with no labels): solutions consist of 3000 μl of 1.34×10^{-6} M of chromomycin A_3+2 μl×J of 2.09×10^{-3} M (in base pairs) of pBR322 DNA, where J=1—18. b, chromomycin A_3 1.34×10^{-6} M (3000 μl)+100 μl of 2.09×10^{-3} M pBR322 DNA.

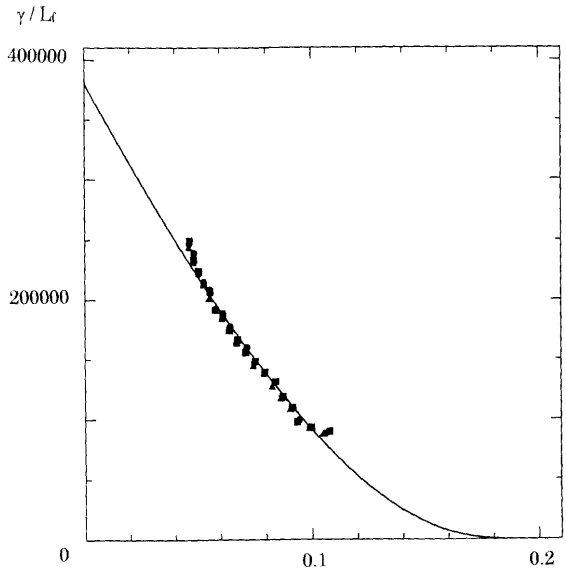

Fig. 5. A Scatchard Plot of Chromomycin A_3 Bound to Plasmid pBR322 DNA

(●): The concentrations of the bound and free drugs (L_f and L_b, respectively) were determined from the fluorescence intensity measurements at 550 nm. (■): from the measurements at 555 nm. (▲): from the measurements at 560 nm. Solid line shows a theoretical curve on the assumption that the equilibrium constant $K=3.8\times10^5$ M^{-1} and number of base pairs occupied by one drug molecule $n^*=0.2$.

plot when it was assumed that $K=3.8\times10^5$ M^{-1} and $n=5.0$.

These values of parameters could be almost uniquely determined; their uncertainties estimated from the experimental errors are as follows:

$K=(3.8\pm0.3)\times10^5$ and $n=5.0\pm0.3$
(or $n^*=0.20\pm0.01$, where $n^*=1/n$, *vide infra*).

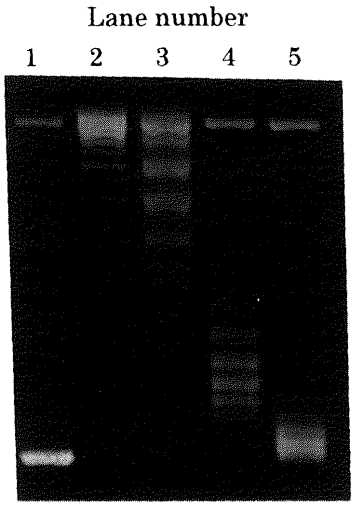

Fig. 6 Electrophoresis Analysis of Topoisomers of pBR322 DNA, Produced by Chromomycine Binding and Topoisomerase Relaxation, Followed by the Removal of the Drug and Enzyme

1, Purified intact pBR322 DNA. 2, completely relaxed pBR322 DNA. No drug was added. 3—5, chromomycin had been added before topoisomerase was introduced. DNA concentration=1.77×10^{-6} M; chromomycin concentrations: 3, 5.60×10^{-7} M; 4, 1.06×10^{-6} M; 5, 1.62×10^{-6} M.

Table 1. Angle of Unwinding of DNA Double Helix Caused by Chromomycin A_3 Binding as Derived from Writhing Number and Equilibrium Constant

Sample No.	C_T [a]	C_B [b]	m [c]	τ_1 [d]	τ_2 [e]	$\Delta\tau$ [f]	ϕ [g]
2	0	0	0	+3—−2	+8—+12		
3	5.60×10^{-7}	4.75×10^{-7}	136	0—−5	+4—+8	4	10.6
4	1.06×10^{-6}	8.92×10^{-7}	256	−6—−11	+2—−2	9	12.6
5	1.62×10^{-6}	1.36×10^{-6}	390	−13—	−1—−5	13	12.0

a) C_T is the total concentration of chromomycin A_3 (M). *b*) C_B is the concentration of chromomycin A_3 bound to DNA (M). *c*) m is the number of chromomycin A_3 molecules bound to one pBR322 (closed circular DNA duplex) molecule. *d*) τ_1 is the writhing number of pBR322 DNA, which comes from the topoisomerase+pBR322 DNA solution containing a given amount of chromomycin A_3, shown in the agarose gel without chloroquine. *e*) τ_2 is the writhing number of the same pBR322 DNA sample as in *d*), shown in the agarose gel with 0.5 μg/ml chloroquine. *f*) $\Delta\tau$ is the change of writhing number of pBR322 DNA caused by the chromomycin A_3 binding. *g*) ϕ is the unwinding angle of pBR322 DNA due to one chromomycin A_3 molecule, calculated by Eq. 3.

Figure 6 shows the results of our electrophoresis experiments. As may be read from the bands in lane 1, the purified native pBR322 DNA was found to be highly super-coiled, containing a small amount of relaxed circular duplex (probably nicked). The supercoiled pBR322 DNA was completely relaxed by the action of topoisomerase I (see lane 2, Fig. 6). The appearance of several bands around $\tau=0$ is understandable as a results of Boltzmann distribution of the relaxed pBR322 DNA at 20 °C in TBE buffer.[1] The sample No. 3 came from the topoisomerase action on the solution containing 5.60×10^{-7} M chromomycin and 1.77×10^{-6} M DNA (in buffer-T, at 37 °C). Because the equilibrium constant K is now known to be 3.8×10^5 M, the concentration of the bound drug in this solution can be calculated as 4.75×10^{-7} M. This means that the number (m) of drug molecules bound per one pBR322 DNA molecule is $m=136$ (Table 1). If, from the electrophoresis analysis, the change of the τ-value ($\Delta\tau$) caused by this drug binding can be determined, the unwinding angle ϕ due to one drug molecule could be calculated by Eq. 3.

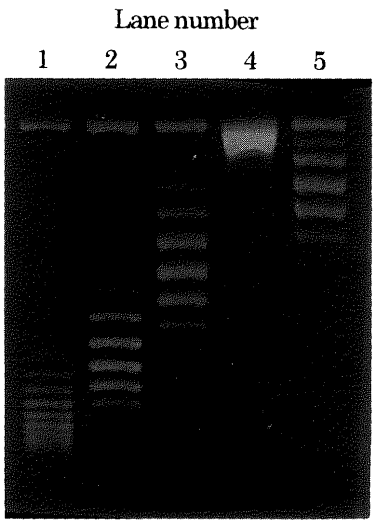

Fig. 7. Electrophoresis Analysis of Topoisomers of pBR322 DNA in a 1% Agarose Gel Containing 0.5 μg/ml of Chloroquine

The samples 1—5 here are the same respectively with the samples 1—5 described in the caption of Fig. 6.

$$\phi = 360\ \Delta\tau/m \text{ degrees} \quad (3)$$

Because of the overlapping of the bands, however, the difference ($\Delta\tau$) of the τ value between samples 2 and 3 is not easily read in our electrophoresis experiment shown in Fig. 6. To overcome this difficulty, the same set of samples (No. 2, 3) was subjected to an electrophoresis experiment in an agarose gel containing 0.5 μg/ml of chloroquine. As may be seen in Fig. 7, the $\Delta\tau$ value between samples 2 and 3 is now determined to be 4. Thus, the unwinding angle ϕ is calculated, from Eq. 3, to be 10.6 degrees (last column of Table 1). Similar experiments were repeated by increasing the concentration of chromomycin A_3 stepwise. As seen in Figs. 6 and 7, the $\Delta\tau$ value also increased stepwise. The calculated m value also rose, but the calculated unwinding angle ϕ remained around 12° (Table 1). Thus, our conclusion is that one molecule of chromomycin A_3 bound to DNA duplex caused an unwinding of the helix by 11.8±1.1 degrees.

The results of our ITC are shown in Fig. 8. For each injection of the drug solution into the DNA solution in the ITC cell, the heat release was measured. The heat of DNA–drug interaction was estimated by subtracting the heat of mere dilution from it. The interaction heat Q has been found to change steeply by an amplitude of 7 kcal/mole on going from a drug/DNA mole ratio=0 to 0.5. Such data should contain information about the binding constant K, the number of the drug sites on one base pair of DNA duplex n^*, and the molar heat of drug–DNA binding ΔH. Here $n^* = 1/n$. An analysis was made by the use of a software "Origin", which was provided by MicroCal, Inc.

By assuming a set of identical sites on DNA duplex, the following equation was used:

$$Q = \frac{n^* M_t \Delta H V_0}{2}\left[1 + \frac{X_t}{n^* M_t} + \frac{1}{n^* K M_t} - \sqrt{\left(1 + \frac{X_t}{n^* M_t} + \frac{1}{n^* K M_t}\right)^2 - \frac{4X_t}{n^* M_t}}\right] \quad (4)$$

where V_0 is the cell volume, M_t is the bulk concentration of DNA base pair, and X_t is the bulk concentration of chromomycin A_3. Incidentally, this software uses n^* instead of n in the McGhee–von Hippel equation (eq.1, *vide supra*). Here, $n^* = 1/n$ from its definition; the number of the drug sites on one base pair of DNA duplex is equal to the reciprocal of the number of base pairs occupied by one drug molecule bound to DNA duplex. Using the observed data, the values of the parameters, n^*, K, and ΔH, were obtained on the software. They were

$$n^* = 0.211, \quad K = 7.32\times10^4\ \mathrm{M}^{-1}, \quad \text{and } \Delta H = -7600\ \text{cal/mol}.$$

As shown in Fig. 8, the calculated curve on the basis of this set of parameter values fit well the observed points. Thus, the parameter values are considered to be significant.

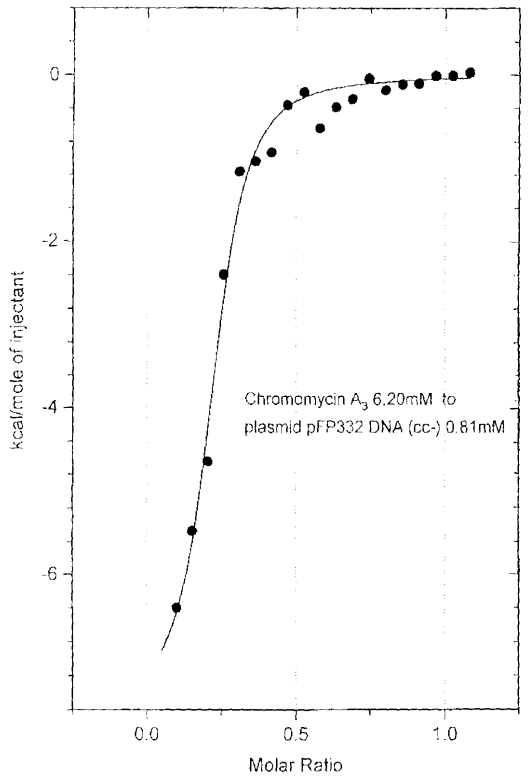

Fig. 8. Plot of the Data of the ITC

To 0.81 mM solution of pFR332 DNA, 6.20 mM solution of chromomycin A_3 was injected at 37 °C. For each injection (9.003 μl), heat of dilution was automatically measured and plotted against the amount of DNA added. The points are experimental and the solid line corresponds to the best-fit curve obtained by least-squared deconvolution. The best values of parameters are 0.211 for n^*, $7.32\times10^4\ \mathrm{M}^{-1}$ for K, and −7600 cal/mol for ΔH^0.

Discussion

It has now been established that chromomycin A_3 binding causes an unwinding of DNA duplex, and that the unwinding angle is 11.8±1.1 degrees. It is interesting that this unwinding angle is nearly equal to that caused by daunomycin.[1] This fact suggests that the chromomycin A_3 binding involves an intercalation as does[9] that of daunomycin. Both from our fluorescence and calorimetric studies, it was concluded that the binding site of this drug corresponds to five contiguous base pairs of the duplex. This is greater than the site (4.0 base pairs) of aclacinomycin A, and this is understandable because chromomycin A_3 has five sugar rings whereas aclacinomycin A has only three.

In comparison with the finding of Gao and Patel,[5] the re-

Table 2. Equilibrium Constant K of the Chromomycin–DNA Binding Reaction in Various Solvents

DNA	Solvent	Temperature (°C)	K (M^{-1})	n	Reference
Calf thymus	0.05 M glycine buffer, pH=9.8 0.1 M EDTA 0.05 M NaCl 0.1 mM $MgCl_2$		2×10^5	5.3	10
Calf thymus	20 mM Tris–HCl, pH=8.0 0.14—21.0 mM Mg^{2+}	25	$(1.0—3.0)\times10^5$	2.8—4.5	11
Poly (dG-dC) ·poly (dG-dC)	20 mM Tris–HCl, pH=8.0 0.33 mM Mg^{2+}	37	2.7×10^5	4.3	12
Poly (dG-dC) ·poly (dG-dC)	20 mM Tris–HCl, pH=8.0 11.3 mM Mg^{2+}	37	3.0×10^5	3.1	12
Poly dG ·poly dC	20 mM Tris–HCl, pH=8.0 0.33 mM Mg^{2+}	37	7.8×10^5	4.8	12
Poly dG ·poly dC	20 mM Tris–HCl, pH=8.0 11.3 mM Mg^{2+}	37	4.6×10^5	6.7	12
pBR322	35 mM Tris–HCl, pH=8.0 72 mM KCl 5 mM $MgCl_2$ 5 mM DTT 5 mM spermidine 0.01% BSA	37	3.8×10^5	5.0	Present study
pFP322	35 mM Tris–HCl, pH=8.0 72 mM KCl 5 mM $MgCl_2$ 9% methanol	37	7.32×10^4	4.7	Present study

sults of our present study suggest that a local mode of ligand–DNA interaction realized in an oligonucleotide may in general be extrapolated into an overall interaction of the same ligand with a long DNA duplex. For the particular chromomycin–DNA case, Gao and Patel[5] demonstrated that a B-to-A conformational transition is required for the octamer duplex to accommodate the chromomycin dimer. This transformation transition is required for the octamer duplex to accommodate the chromomycin dimer. This transformation must be accompanied by a duplex unwinding of 23°, because A-helical conformation has 360/11=32.7° residual rotation (around the helix axis) whereas B-helical conformation has 360/10=36°, so that $(32.7-36)\times7=-23°$. This mean that in their model $\phi=-11.5°$ and $n=4$. This set of parameters is in a close agreement with what we found for the long DNA duplex (pBR322); $\phi=11.8\pm1.1°$ and $n=5.0\pm0.3$.

The equilibrium constant K (Eq. 2) of the chromomycin–DNA binding reaction has been determined by several previous investigators under somewhat different conditions.[10—12] They are listed in Table 2, along with what we have determined in the present study. In every case, the solvent involves 0.1 mM or more Mg^{2+}, and the K value remains nearly constant around $2—4\times10^5\ M^{-1}$. When 9% methanol is added to the solvent, however, K becomes much lower; $K=7.32\times10^4\ M^{-1}$ (last line of Table 2). In this solvent, where the solubility of chromomycin is sufficiently high, a thermodynamic characterization of the chromomycin–DNA interaction could be made. The chromomycin–DNA binding has been found to be an exothermic reaction, with an enthalpy change of $\Delta H=-7600$ cal/mol$=-31.82$ kJ/mol. At 37 °C, the free energy change ΔG is calculated from $K=7.32\times10^4\ M^{-1}$ as $\Delta G=-6897$ cal/mol$=-28.9$ kJ/mol. Therefore, the entropy change must be $\Delta S=-9.5$ J mole^{-1} K^{-1}. It is interesting that the entropy change is negative here, in contrast to the case of daunomycin–DNA interaction, where ΔS is positive.[13] A ligand–DNA binding, in general, causes a quenching of the molecular movement. The overall molecular translational freedom is reduced and probably the conformational flexibility becomes smaller. On the basis of this particular context, the negative ΔS for the chromomycin–DNA interaction is understandable. If a ligand–DNA binding involves a great amount of hydrophobic interaction, on the other hand, this may overcome the above effect to make ΔS positive. The daunomycin–DNA binding may correspond to this latter case.

References

1) Utsuno K., Tsuboi M., *Chem. Pharm. Bull.*, **45**, 1551—1557 (1997).
2) Hayasaka T., Inoue Y., *Biochemistry*, **8**, 2342—2347 (1969).
3) Miyamoto M., Kawamatsu Y., Kawashima K., Shinohara M., Tanaka K., Tatsuoka S., Nakanishi K., *Tetrahedron*, **23**, 421—437 (1967).
4) Baguley B. C., *Mol. Cell. Biochem.*, **43**, 167—181 (1982).
5) Gao X., Patel D. J., *Biochemistry*, **28**, 751—762 (1989).
6) Liu C., Chen F.-M., *Biochemistry*, **33**, 1419—1424 (1994).
7) Scatchard G., *Ann. New York Acad. Sci.*, **51**, 660—672 (1949).
8) McGhee J. D., von Hippel P. H., *J. Mol. Biol.*, **86**, 469—489 (1974).
9) Wang A. H.-J., Ugehetto G., Quigley G. J., Rich A., *Biochemistry*, **26**, 1152—1163 (1987).
10) Behr W., Honikel K., Hartmann G., *Eur. J. Biochem.*, **9**, 82—92 (1969).
11) Aich P., Sen R., Dasgupta D., *Biochemistry*, **31**, 2988—2997 (1992).
12) Majee S., Sen R., Guha S., Bhattacharyya D., Dasgupta D., *Biochemistry*, **36**, 2291—2299 (1997).
13) Remeta D. P., Mudd C. P., Berger R. L., Breslauer K. J., *Biochemistry*, **32**, 5064—5073 (1993).