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Bleomycin A_5 is analogous in mechanism to a DNA-cleaving enzyme: a microcalorimetric study

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Abstract

Microcalorimetry, UV–VIS spectroscopy and HPLC were used to conduct thermodynamic and kinetic investigations of the scission of calf thymus DNA catalyzed by bleomycin A_5 (BLM-A_5) in the presence of ferrous ion and oxygen. The molar reaction enthalpy for the cleavage, the Michaelis constant for calf thymus DNA and the turnover number of BLM-A_5 were calculated by a novel thermokinetic method for enzyme-catalyzed reactions to be -577 ± 19 kJ mol^{-1}, $(2.04 \pm 0.38) \times 10^{-5}$ mol dm^{-3} and $(2.28 \pm 0.49) \times 10^{-2}$ s^{-1}, respectively, at 37.00 °C. This DNA cleavage was a fast and exothermic reaction. The catalytic efficiencies of BLM-A_5 and of some DNA-cleaving enzymes, such as *Taq*I restriction endonuclease and *Nae*I endonuclease, are of the same order of magnitude. By comparing the molar enthalpy change for the cleavage of calf thymus DNA induced by BLM-A_5 with those for the scission of calf thymus DNA mediated by adriamycin and by (1, 10-phenanthroline)-copper, it was found that BLM-A_5 possessed the highest DNA cleavage efficiency among these DNA-damaging agents. The UV–VIS spectral experiments showed that BLM-A_5 itself remained unchanged chemically after the cleavage. The products of this scission, including four kinds of free nucleic acid bases, were separated and detected by HPLC. These results strongly suggest that BLM-A_5 is analogous in mechanism to a DNA-cleaving enzyme, although it is much smaller than such type of enzymes. A possible mechanism for the cleavage of DNA catalyzed by BLM was suggested in this paper.

Keywords: Bleomycin; DNA-cleaving enzyme; Kinetics; Microcalorimetry; Thermodynamics

1. Introduction

The bleomycins (BLMs, Fig. 1) are a family of naturally occurring, structurally related, glycopeptide-derived antitumor antibiotics discovered by Umezawa et al. from cultures of *Streptomyces verticillus* in 1966 [1], which have more than 200 members, such as A_2, A_5 and B_2 [2]. BLMs consist of an unusual linear hexapeptide, a disaccharide and a terminal amine (the R group in Fig. 1). Mixtures of BLMs are presently used for the clinical treatment of a variety of cancers, notably squamous cell carcinomas, testicular tumors and non-Hodgkin's lymphoma [2]. The therapeutic effect of BLM is believed to result from its ability to induce single- and double-strand breakage of DNA

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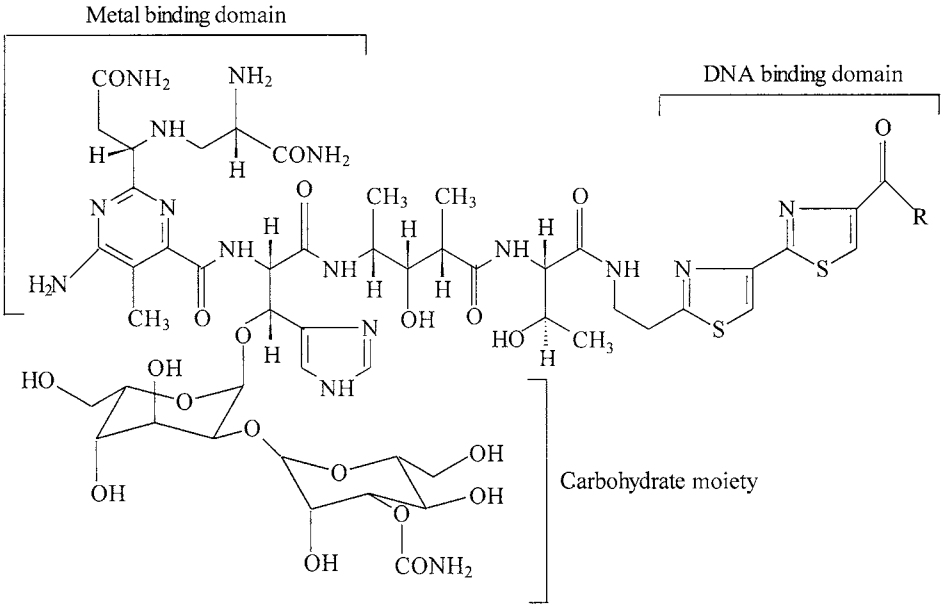

BLM-A_2: R = $NH(CH_2)_3S^+(CH_3)_2$
BLM-A_5: R = $NH(CH_2)_3NH(CH_2)_4NH_2$
BLM-B_2: R = $NH(CH_2)_4NHC(=NH)NH_2$

Fig. 1. Structure of BLM-A_2, A_5 and B_2.

molecules by oxidation of the deoxyribose moiety in the presence of oxygen and a redox-active metal ion, e.g. Fe and Co [2–6]. On the other hand, RNA is also considered as a therapeutically relevant target for BLM [7,8]. It is found that the BLM-induced autoxidation of ferrous iron follows Michaelis–Menten kinetics which are characteristic of enzymatic reactions [9,10]. Although a significant number of experimental approaches have been used to elucidate the mechanism of DNA cleavage by BLM in the past two decades [2–6,11–18], both thermodynamic and kinetic information for the scission, which is necessary for a thorough understanding of the mechanism, is eagerly awaited. The purpose of this investigation is to provide detailed thermodynamic and kinetic data for BLM-mediated DNA degradation to furnish insights into the anticancer mechanism of BLM.

Microcalorimetry is an important tool for the study of both thermodynamic and kinetic properties of biological macromolecules by virtue of its general applicability, high accuracy and precision [19–22]. Recently, this method has yielded a large amount of data on the binding reactions of DNA with DNA-targeting molecules, such as adriamycin (ADM) [23], daunomycin [23,24], Hoechst 33258 [25], ethidium bromide [26], 2,7-diazapyrene [27] and dodecyl trimethylammonium bromide [28]. Only a limited number of authors, however, have paid attention to the energetics of drug-induced cleavage of DNA [29,30].

In a previous publication from this laboratory [30], microcalorimetry was used to measure the change in enthalpy for the scission of calf thymus DNA induced by ADM in the presence of ferric ion, vitamin C (Vc) and oxygen. In the present paper, microcalorimetry, UV–VIS spectroscopy and HPLC were combined to study the scission of calf thymus DNA by a mixture of bleomycin A_5 (BLM-A_5), ferrous iron and oxygen. A novel thermokinetic method for an enzyme-catalyzed reaction was proposed and employed to produce not

only the thermodynamic constant ($\Delta_r H_m$) but also the kinetic properties (K_m and k_2) of the cleavage of DNA catalyzed by BLM-A_5 with the result that BLM-A_5 is analogous in mechanism to a DNA-cleaving enzyme. In order to gain insights into the nucleotide binding interactions of BLM, we have elucidated the binding constant (K_b) and the standard thermodynamic parameters ($\Delta_b H_m^\circ$, $\Delta_b G_m^\circ$ and $\Delta_b S_m^\circ$) for the binding of BLM-A_5 to calf thymus DNA using microcalorimetry. The results help understand the binding mode of BLM-A_5 to DNA during the cleavage of DNA catalyzed by BLM-A_5.

2. Theory and method

For a simple single-substrate, single-intermediate, enzyme-catalyzed reaction occurring in a batch reactor (BR) with negligible mass-transfer limitations, from Michaelis–Menten kinetics, it follows that:

$$-\frac{1}{t}\ln(1-x) = \frac{k_2[E]_0}{K_m} - \frac{[S]_0}{K_m}\left(\frac{x}{t}\right) \tag{1}$$

where x is the fraction of substrate converted into product at time t, which is non-dimensional, K_m the Michaelis constant, $[S]_0$ and $[E]_0$ the initial concentrations of substrate and enzyme in reacting systems, respectively, and k_2 also known as the turnover number of the enzyme [31], the rate constant of breakdown of the enzyme–substrate complex to product.

If the heat-transfer process in a BR obeys Tian's equation [19,32–34], the substrate conversion x at time t in a BR may be written respectively as

$$x = \frac{\Delta + ka}{kA} \tag{2}$$

where Δ is the peak height at time t, a the peak area before time t, A the total area under the calorimetric curve, and k is the Newton cooling constant of the calorimeter system which can be easily determined by electric calibration [33].

According to the thermo-analytical analog curve method [35], the calorimetric curve for a reaction occurring in a conduction calorimeter can be approximately simulated by the following relationship:

$$\Delta = \alpha t\, e^{-k\beta t} \tag{3}$$

At $t = t_m$, $d\Delta/dt = 0$, and $\Delta = \Delta_m$, substituting in Eq. (3), we get

$$\alpha = \frac{e\,\Delta_m}{t_m} \tag{4}$$

$$\beta = \frac{1}{kt_m} \tag{5}$$

where α and β are the analog parameters related to the thermokinetic system, Δ_m and t_m are the calorimetric curve characteristic data representing the maximum calorimetric height and time corresponding to Δ_m, respectively. For a fast reaction, the value of β turns out to be 1. For a slow reaction, however, the value of β is 2/3 [35].

Combining Eqs. (3)–(5), we get

$$\Delta = \frac{t}{t_m}\Delta_m\, e^{\frac{1-t}{t_m}} \tag{6}$$

$$k = \frac{e\,\Delta_m}{\beta A} \tag{7}$$

Substituting Eq. (7) in Eq. (2), we obtain

$$x = \frac{a}{A} + \frac{\beta\Delta}{e\,\Delta_m} \tag{8}$$

When a single-substrate enzyme-catalyzed reaction is taking place in a conduction calorimeter, the molar reaction enthalpy is

$$\Delta_r H_m = \frac{Q_{1,\infty}}{V_T[S]_0} \tag{9}$$

where $Q_{1,\infty}$ is the total heat effect of reaction which can be calculated by the integration type of Tian's equation from the experimental calorimetric curves, and V_T the total volume of the reacting system (in this paper, $V_T = 6.00\ cm^3$).

Eqs. (1), (8) and (9) are called the analog calorimetric curve model of a single-substrate enzyme-catalyzed reaction. It is a novel application of the thermo-analytical analog curve method, and suitable to both fast and slow enzyme-catalyzed reactions. A plot of $-\ln(1-x)/t$ against x/t is linear with an axis intercept of $k_2[E]_0/K_m$ and a slope of $-[S]_0/K_m$. The values of K_m and k_2 can be calculated from the slope and intercept, respectively, using the calorimetric data from only a single experiment.

3. Experimental

3.1. Reagents

Calf thymus DNA (Sigma Chemical Co., St. Louis, MO, USA) was purified by ethanol precipitation and centrifugal dialysis, and sheared by sonication at ice bath temperatures for 30 min. The absorbances at 260 nm, A_{260}, and at 280 nm, A_{280}, for purified DNA were measured at room temperature. From the results, the ratio of A_{260} to A_{280} is about 2.07. DNA concentrations were determined spectroscopically at 260 nm using a molar extinction coefficient of 13,200 $dm^3\ mol^{-1}\ cm^{-1}$, and expressed as base pair concentrations throughout this paper. The concentration of BLM-A_5 (Hebei Pharmaceutical Factory, Tianjin, China) was determined at 291 nm using a molar extinction coefficient of 15,500 $dm^3\ mol^{-1}\ cm^{-1}$, and a molar extinction coefficient of 11,500 $dm^3\ mol^{-1}\ cm^{-1}$ at 480 nm was used for free ADM (Haimen Pharmaceutical Factory, Zhejiang, China). $FeCl_2{\cdot}4H_2O$ (A.R. grade) (Merck, Germany). Other chemicals used were domestic and of A.R. grade. All reagent solutions were prepared in 0.01 mol dm^{-3} Tris–HCl buffer (pH, 7.4). As the $FeCl_2$ solution is easily oxidized by oxygen, it was placed in a brown bottle and then flushed with purified nitrogen for 10 min, sealed, and stored in a refrigerator until use. Moreover, it was freshly prepared on each occasion.

3.2. Instrumentation

The cleavage of calf thymus DNA by a mixture of BLM-A_5, ferrous ion and oxygen, and the binding of BLM-A_5 to calf thymus DNA, were studied in 0.01 mol dm^{-3} Tris–HCl buffer at pH 7.4 and 37.00 °C. The heat effects of the reactions mentioned above were determined at 37.00 °C using a LKB-2107 batch microcalorimeter (Stockholm, Sweden), which consists of a batch reactor with a heat-conduction isothermal calorimeter. The calorimeter was designed by Wadsö in 1968 [36], and is no longer manufactured and has been replaced by the thermometric TAM. There are two compartments to each batch vessel separated by a weir [36]. For the experiments on DNA cleavage, compartment I of the reaction vessel contained 2.00 cm^3 of a $FeCl_2$ solution, and compartment II of the reaction vessel contained 4.00 cm^3 of a DNA–BLM–O_2 mixture. This multi-component system was prepared by mixing DNA and BLM-A_5 solutions and saturated with purified oxygen before calorimetric experiments. To avoid the re-oxidation of $FeCl_2$ solution on exposure to air, purified N_2 was passed into one compartment of the vessel while sample was added to the other. As soon as samples were added, the source of N_2 was removed, and the plug for the reaction vessel was closed tightly at once. The same procedure was used for adding samples to the reference vessel. For the experiments on DNA binding, compartment I of the reaction vessel contained 2.00 cm^3 of a DNA solution, and compartment II of the reaction vessel contained 4.00 cm^3 of a BLM-A_5 solution. To avoid the influence of the heat effects of diluting and mixing, etc. on the results, the contents and quantities in both vessels were as close as possible except that DNA was not added to the reference vessel. The heat released by dilution of DNA is negligible.

UV–VIS spectra were measured using a Shimadzu UV-300 spectrophotometer. Two samples, one of which is 2.15×10^{-5} mol dm^{-3} BLM-A_5 and the other a reaction mixture containing 2.15×10^{-5} mol dm^{-3} BLM-A_5, 2.00×10^{-5} mol dm^{-3} ferrous iron and 1.515×10^{-5} mol dm^{-3} calf thymus DNA, were chosen. The solutions were saturated with purified oxygen and incubated in 0.05 mol dm^{-3} Tris–HCl buffer at pH 7.4 and 20 °C for 30 min, and then scanned from 250 to 500 nm.

A Hitachi-635A HPLC equipped with a UV detector set at the wavelength $\lambda = 254$ nm and a reverse-phase C-18 column (4 mm i.d. × 30 cm long, particle diameter about 5 μm) thermostated at 20 °C were used for the analysis of the products of the DNA cleavage induced by BLM-A_5. The mobile phase used in the gradient elution consisted of (I) 0.01 mol dm^{-3} KH_2PO_4 solution adjusted to pH 5.52 with KOH and (II) CH_3OH:H_2O (80:20 v/v). The flow rate was 1 $cm^3\ min^{-1}$. A reaction mixture, which contained 6.4×10^{-5} mol dm^{-3} BLM-A_5, 2×10^{-3} mol dm^{-3} ferrous iron and 10^{-3} mol dm^{-3} calf thymus DNA, was saturated with purified oxygen, incubated at 20 °C for 24 h, and then precipitated by ethanol. The precipitate was removed by a filter (0.25 μm), and the absorbance of the upper phase was measured at 254 nm. The concentration of four nucleic acid bases in the standard base solution, including adenine

(A), guanine (G), cytosine (C) and thymine (T), was 5×10^{-4} mol dm^{-3}.

4. Results

4.1. Thermodynamics and kinetics of the cleavage of DNA catalyzed by BLM-A_5

As shown in Fig. 2, the cleavage of calf thymus DNA by a mixture of BLM-A_5, ferrous ion and oxygen is a fast reaction and the experimental calorimetric curve can be reasonably well fitted by the analog calorimetric curve between 75 and 210 s at 37.00 °C. Therefore, the calorimetric data in this range have been chosen to analyze the thermodynamic and kinetic parameters for the scission by means of the analog calorimetric curve method. The substrate conversion x at time t in one experiment on the DNA cleavage by BLM-A_5 can be calculated by Eq. (8) from the calorimetric data shown in Fig. 2. A plot of $-\ln(1-x)/t$ against x/t is linear with the value of y-axis intercept being 1.323×10^{-2} s^{-1}, the value of slope being -0.7342, and the linear correlation coefficient being -0.9967. Then, the values of K_m and k_2 can be calculated from the slope and intercept to be 23.6 μM and 2.90×10^{-2} s^{-1}, respectively. It should be pointed out that Fe^{2+} is used in about 30-fold molar excess relative to BLM in spite of the fact that only about three turnovers (presumably corresponding to DNA cleavage events) are observed within the first critical 100 s. The excess free Fe^{2+} is oxidized to Fe^{3+} in the presence of oxygen, which is catalyzed by BLM with 5000 turnovers min^{-1} [9].

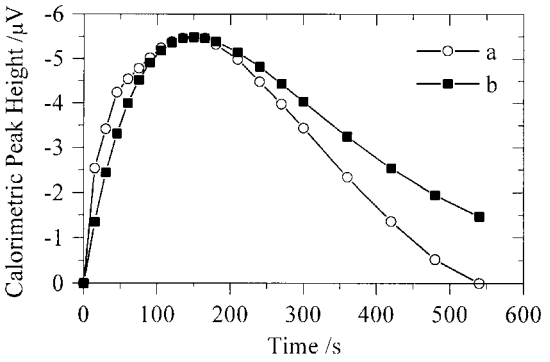

Fig. 2. Experimental calorimetric (a) and the corresponding analog calorimetric (b) of the scission of calf thymus DNA by a mixture of BLM-A_5, Fe^{2+} and O_2 at 37.00 °C. For curve b, $\Delta = 0.03653t\,e^{\frac{1-t}{150}}$, and $\beta = 1$. The initial concentrations of calf thymus DNA, BLM-A_5, Fe^{2+} and O_2 are 1.733×10^{-5}, 1.075×10^{-5}, 3.383×10^{-4} and 6.54×10^{-4} mol dm^{-3}, respectively.

Table 1 summarizes the molar reaction enthalpies ($\Delta_r H_m$) and the kinetic parameters (K_m and k_2) for the cleavage of calf thymus DNA by a mixture of BLM-A_5, Fe^{2+} and O_2 at different DNA concentrations and at 37.00 °C. As we can see from Table 1, the relative standard deviations for these thermodynamic and kinetic data, $\Delta_r H_m$, K_m and k_2, are small, and the correlation coefficient of the model approximates to -1. These results show that the analog calorimetric curve model proposed herein is suitable for describing both thermodynamic and kinetic properties of the

Table 1
Thermodynamic and kinetic data of the cleavage of calf thymus DNA by a mixture of BLM-A_5, Fe^{2+} and O_2 at 37.00 °C[a]

No.	$C_{DNA,0} \times 10^5$ (mol dm^{-3})	$C_{FeCl_2,0} \times 10^4$ (mol dm^{-3})	$C_{O_2,0} \times 10^4$ (mol dm^{-3})	$-Q_{1,\infty}$ (mJ)	R[b]	$-\Delta_r H_m$ (kJ mol^{-1})	$K_m \times 10^5$ (mol dm^{-3})	$k_2 \times 10^2$ (s^{-1})
1	0.3465	3.383	6.58	12.00	−0.9916	577	2.41	1.77
2	0.6930	3.383	6.57	25.23	−0.9968	607	2.19	1.83
3	0.6930	6.767	6.57	23.28	−0.9931	560	1.57	1.75
4	1.733	3.383	6.54	59.65	−0.9967	574	2.36	2.90
5	1.733	3.383	6.54	62.62	−0.9912	602	2.33	2.21
6	1.733	6.767	6.54	59.50	−0.9970	572	1.48	2.30
7	3.465	3.383	6.50	115.5	−0.9957	556	2.24	2.49
8	3.465	6.767	6.50	117.3	−0.9989	564	1.70	2.98
						577 ± 19	2.04 ± 0.38	2.28 ± 0.49

[a] $C_{BLM\text{-}A_5,0} = 1.075 \times 10^{-5}$ mol dm^{-3}. The molar enthalpy change for the reaction of BLM-A_5, Fe^{2+} and O_2 has been determined by microcalorimetry to be -34.4 ± 3.2 kJ mol^{-1}. Data are expressed as mean ± S.D.

[b] R is the correlation coefficient of $-\ln(1-x)/t$ correlating with x/t.

DNA scission by BLM. From Fig. 2 and Table 1, it can also be seen that this DNA cleavage was a fast and exothermic reaction and followed the Michaelis–Menten kinetics. This suggests BLM-A_5 is unlike a chemical catalyst but analogous in mechanism to an enzyme. Thus, the observed rate law for the cleavage of DNA catalyzed by BLM-A_5 at excessive ferrous ion and oxygen concentrations can be expressed as follows:

$$\upsilon = \frac{k_2 C_{\mathrm{BLM},0} C_{\mathrm{DNA},0}}{K_\mathrm{m} + C_{\mathrm{DNA},0}} \tag{10}$$

As can be seen in Table 1, the value of K_m is approximately inversely proportional to the concentration of Fe^{2+}, suggesting that the excess free Fe^{2+} is likely to bind to the DNA substrate and may well alter the observed kinetics as well.

4.2. UV–VIS spectra of BLM-A_5

Fig. 3 shows the UV–VIS spectra from 250 to 500 nm of BLM-A_5 before and after the cleavage of calf thymus DNA by a mixture of BLM-A_5, Fe^{2+} and O_2. It can be seen from Fig. 3 that the large underlying peak at 291 nm for BLM, which has been ascribed to the bithiazole π–π^* and n–π^* transitions [17], does not shift after this scission. This result showed that BLM-A_5 itself remained unchanged chemically at the end of the cleavage of calf thymus DNA by a mixture of BLM-A_5, Fe^{2+} and O_2, suggesting BLM-A_5 is analogous in mechanism to an enzyme. The band between 370 and 400 nm in Fig. 3 may result from charge transfer transitions between the iron atom and BLM [17].

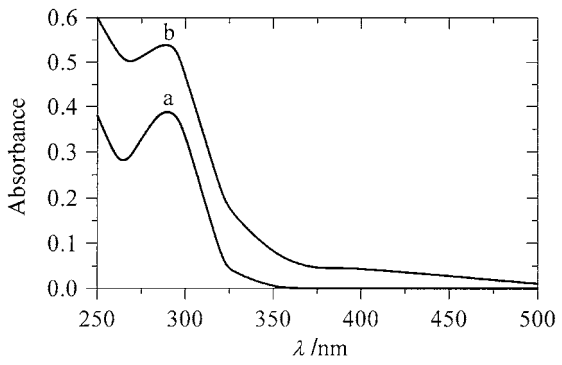

Fig. 3. UV–VIS spectra of BLM-A_5 before and after the cleavage of calf thymus DNA by a mixture of BLM-A_5, Fe^{2+} and O_2. (a) 2.15×10^{-5} mol dm^{-3} BLM-A_5; (b) a reaction mixture containing 2.15×10^{-5} mol dm^{-3} BLM-A_5, 2.00×10^{-5} mol dm^{-3} Fe^{2+} and 1.515×10^{-5} mol dm^{-3} calf thymus DNA, after saturation with O_2 and incubation in 0.05 mol dm^{-3} Tris–HCl buffer at pH 7.4 and 20 °C for 30 min.

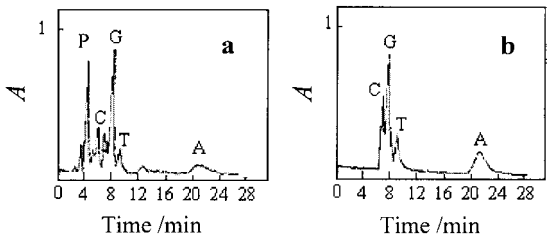

Fig. 4. (a) HPLC profile of the products produced when calf thymus DNA is incubated with BLM-A_5, Fe^{2+} and O_2. (b) Standard HPLC profile of four nucleic acid bases, including adenine (A), guanine (G), cytosine (C) and thymine (T).

4.3. HPLC profile of the products for the DNA cleavage catalyzed by BLM-A_5

Fig. 4a shows the HPLC profile of the products produced when calf thymus DNA is incubated with BLM-A_5, Fe^{2+} and O_2, and Fig. 4b displays the standard HPLC profile of four nucleic acid bases, including adenine (A), guanine (G), cytosine (C) and thymine (T). By comparison of these HPLC profiles, it is clear that the products of this scission included four kinds of free nucleic acid bases, namely A, G, C and T. Peak P in Fig. 4a may be C-4′ hydroxyapurinic acid [12], which needs to be identified further.

4.4. Thermodynamics of the binding of BLM-A_5 to DNA

In order to gain insights into the nucleotide binding interactions of BLM, we have characterized the thermodynamic parameters for BLM-A_5 binding to calf thymus DNA by using microcalorimetry. Combining the thermodynamic model for the binding of small molecules to DNA [30], we reported the intrinsic binding constant (K_b) and the standard thermodynamic parameters ($\Delta_\mathrm{b}H^\circ_\mathrm{m}$, $\Delta_\mathrm{b}G^\circ_\mathrm{m}$ and $\Delta_\mathrm{b}S^\circ_\mathrm{m}$) of this binding reaction for the first time.

Fig. 5 shows two calorimetric curves of BLM-A_5 binding to calf thymus DNA. Table 2 summarizes the

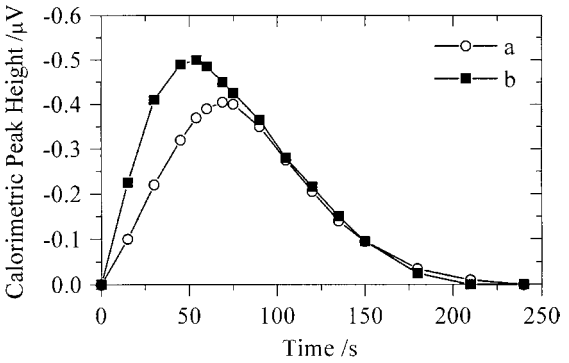

Fig. 5. Calorimetric curves of BLM-A_5 binding to calf thymus DNA at 37.00 °C. The initial concentration of calf thymus DNA was 1.386×10^{-4} mol dm^{-3} and the initial concentrations of BLM-A_5 were (a) 4.300×10^{-5} mol dm^{-3} and (b) 8.600×10^{-5} mol dm^{-3}, respectively.

thermodynamic data for this binding reaction at 37.00 °C, in which the values of K_b, $\Delta_b H^\circ_m$ and n are obtained by fitting the experimental data (the empty circles in Fig. 6A) to a thermodynamic model as described [30]. The solid line in Fig. 6A, the apparent molar enthalpy change for this binding as predicted by the parameters in Table 2, accords well with the experimental data. As can be seen in Table 2, the relative standard deviations for these thermodynamic data are small, indicating that the thermodynamic model for the binding of small molecules to DNA [30], is reasonable.

4.5. Thermodynamics of the binding of ADM and (1,10-phenanthroline)-copper to DNA

To establish the action mode of BLM-A_5 to DNA, we investigated the energetics for both the binding reactions of ADM and (1,10-phenanthroline)-copper to calf thymus DNA, and found that their thermodynamic

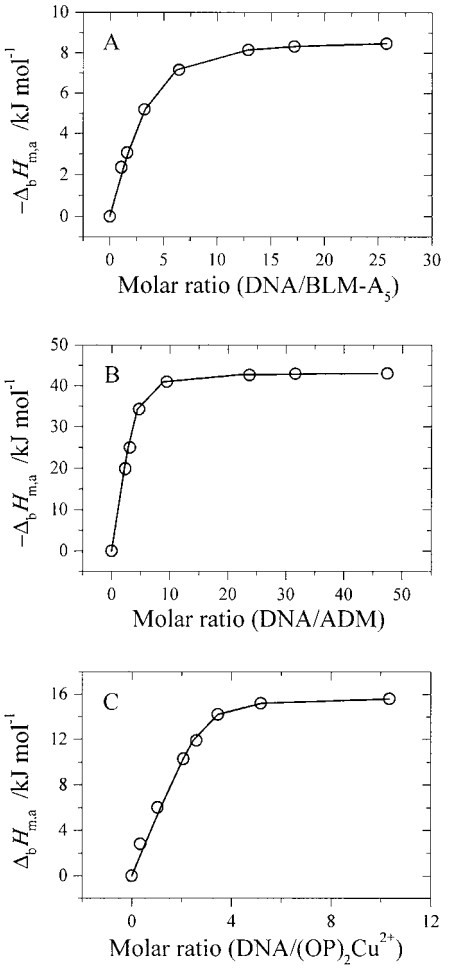

Fig. 6. Apparent molar enthalpy changes for the binding reactions of (A) BLM-A_5, (B) ADM and (C) $(OP)_2Cu^{2+}$, to calf thymus DNA at 37.00 °C. The initial concentrations of calf thymus DNA were 1.386×10^{-4} mol dm^{-3} (A and C) and 1.364×10^{-4} mol dm^{-3} (B). Empty circles, experimental data; solid lines, curves predicted by the parameters in Table 2.

Table 2
Thermodynamic parameters for the interactions of three antitumor drugs, BLM-A_5, ADM and (1,10-phenanthroline)-copper, with calf thymus DNA at 37.00 °C[a]

Drugs	$K_b \times 10^{-4}$	n	$\Delta_b H^\circ_m$ (kJ mol^{-1})	$\Delta_b G^\circ_m$ (kJ mol^{-1})	$\Delta_b S^\circ_m$ (J mol^{-1} K^{-1})	Action modes
BLM-A_5	4.19 ± 0.94	5.31 ± 0.12	−10.2 ± 0.4	−27.4 ± 0.6	55.5 ± 3.2	Partial intercalation
ADM	10.9 ± 1.6	4.83 ± 0.92	−46.3 ± 0.9	−29.9 ± 0.4	−52.9 ± 4.2	Intercalation
$(OP)_2Cu^{2+}$	21.6 ± 5.7	3.07 ± 0.10	16.3 ± 0.2	−31.7 ± 0.7	155 ± 3	Groove binding

[a] Data are expressed as mean ± S.D.

binding parameters were different from those of BLM-A_5. ADM is an intercalator, which inserts its aromatic ring between adjacent base pairs of DNA [23,37], and (1,10-phenanthroline)-copper ($(OP)_2Cu^{2+}$) binds to either the major or minor groove of the double helix [38]. The thermodynamic data for these binding reactions at 37.00 °C are listed in Table 2. The solid lines in Fig. 6B and 6C are the apparent molar enthalpy changes for these binding reactions as predicted by the parameters in Table 2, and in agreement with the experimental data. The relative standard deviations for these thermodynamic data are small, indicating that the thermodynamic model for the binding of small molecules to DNA [30], is reasonable.

5. Discussion

5.1. BLM-A_5 is analogous in mechanism to a DNA-cleaving enzyme

BLM has three functional domains (Fig. 1). The metal binding domain is required for metal complexation, oxygen binding and activation [6], and corresponds to the catalytic site of DNA-cleaving enzymes, e.g. Eco RI endonuclease [13]. The DNA binding domain, encompassing the bithiazole moiety, can be regarded as the substrate binding site [13]. The carbohydrate moiety is involved in cell permeability and selective tumor recognition [6]. Although BLM is much smaller than ''real'' DNA-cleaving enzymes, it is comparable, both in size and in domains, to the cleft of the active site of such type of enzymes, e.g. Eco RI endonuclease [13].

Table 3 compares the kinetic parameters for BLM-A_5 with those for carbonic anhydrase [39], lysozyme [39], *Taq*I restriction endonuclease [40] and *Nae*I endonuclease [41]. As shown in Table 3, the catalytic efficiency (represented by k_2) of BLM-A_5 is of the same order of magnitude as that of some DNA-cleaving enzymes, such as *Taq*I restriction endonuclease and *Nae*I endonuclease. The substrate binding power (represented approximately by K_m) of BLM-A_5 is definitely comparable to that of most enzymes. Combining the results from the UV–VIS spectral experiments, we suggest that BLM-A_5 is analogous in mechanism to a DNA-cleaving enzyme, although it is much smaller than such type of enzymes. It has been suggested that BLM-A_2 is analogous in mechanism to a ferrous oxidase [9,10]. Therefore, BLM is analogous in mechanism to both a DNA-cleaving enzyme and a ferrous oxidase.

According to Tsou [42], the active site of an enzyme is situated in a limited region more flexible than the enzyme molecule as a whole. The shifting specificity model for enzyme catalysis [39] maintains that the inability of small molecular weight enzyme mimics to both bind a substrate well and catalyze its conversion rapidly, stems from a lack of conformational flexibility of the mimics to bind both ground state and transition state structures efficiently. The kinetic data presented here demonstrate that BLM-A_5 both binds DNA well and catalyze its conversion rapidly, indicating that BLM-A_5 may display conformational flexibility to some extent. Combining the results from the UV–VIS spectral and HPLC experiments, we strongly suggest that BLM-A_5 is analogous in mechanism to a DNA-cleaving enzyme, although it is much smaller than such type of enzymes.

5.2. DNA cleavage efficiency of BLM-A_5

In Table 4, we compare the molar enthalpy change for the cleavage of calf thymus DNA induced by

Table 3
Comparison of the kinetic parameters for BLM-A_5 and those for carbonic anhydrase, lysozyme, *Taq*I restriction endonuclease and *Nae*I endonuclease

Enzymes	Substrates	K_m (mol dm^{-3})	k_2 (s^{-1})	Reference
Carbonic anhydrase	HCO_3^-	9.6×10^{-3}	4×10^5	[39]
Lysozyme	$(NAG)_2$[a]	1.75×10^{-4}	0.5	[39]
*Taq*I restriction endonuclease	DNA	5.3×10^{-8}	2.2×10^{-2}	[40]
*Nae*I endonuclease	DNA	1.0×10^{-8}	4.5×10^{-2}	[41]
BLM-A_5	DNA	2.11×10^{-5}	2.28×10^{-2}	This work

[a] NAG is *N*-acetylglucosamine.

Table 4
Comparison of the molar enthalpy change for the cleavage of calf thymus DNA induced by BLM-A_5 and those for the scission of calf thymus DNA mediated by two DNA-damaging agents, ADM and (1,10-phenanthroline)-copper[a]

Cleavage systems	$\Delta_r H_m$ (kJ mol^{-1})	Products	Reference
BLM-A_5–Fe^{2+}–O_2[b]	−577 ± 20	Free nucleic bases	This work, [2,5,11,12]
ADM–Fe^{3+}–Vc–O_2[c]	−147.1 ± 6.1	DNA fragments	[30,37]
$(OP)_2Cu^{2+}$–ME–O_2[d]	−35.1 ± 1.8	Linear DNA	[29]

[a] Data are expressed as mean ± S.D.
[b] $T = 37.00$ °C; pH 7.4.
[c] $T = 25.00$ °C; pH 7.4.
[d] $T = 37.00$ °C; pH 7.0.

BLM-A_5 with those for the scission of calf thymus DNA mediated by two well-known DNA-damaging agents, ADM [37,43] and (1,10-phenanthroline)-copper [29,38]. Scission of calf thymus DNA induced by BLM in the presence of Fe^{2+} and O_2, converted calf thymus DNA to free nucleic bases [2,5,11,12]. From electrophoresis experiments, it was found that nicking of pBR-322 DNA by a mixture of ADM, Fe^{3+}, Vc and O_2 and by a mixture of (1,10-phenanthroline)-copper(II), 2-mercaptoethanol (ME) and O_2 converted pBR-322 DNA to small DNA fragments [37] and linear DNA [29], respectively. As is seen in Table 4, the higher the degree of DNA strand scission by drugs, the larger the molar enthalpy change for the DNA cleavage. BLM-A_5 possessed the highest DNA cleavage efficiency among these DNA-damaging agents.

5.3. Mechanism for the cleavage of DNA catalyzed by BLM

According to the experimental results in this and other laboratories [2,4,5,11,12,14,16], we proposed a possible mechanism for the cleavage of DNA catalyzed by BLM:

$$\mathrm{BLM} + \mathrm{Fe}^{2+} + \mathrm{O_2} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{activated BLM (rapid equilibrium)} \quad (11)$$

$$\mathrm{DNA} + \text{activated BLM} \underset{k'_{-1}}{\overset{k'_1}{\rightleftharpoons}} \mathrm{DNA} \times \text{activated BLM (rapid equilibrium)} \quad (12)$$

$$\mathrm{DNA} \times \text{activated BLM} \xrightarrow[k_2]{} \text{scission products} + \mathrm{BLM} + \mathrm{Fe}^{3+} + \mathrm{H_2O_2}\ \text{(slow)} \quad (13)$$

Applying the equilibrium approximation to the mechanism above, we can easily prove that

$$\upsilon = \frac{k_2 C_{\mathrm{BLM},0} C_{\mathrm{DNA},0}}{K_m + C_{\mathrm{DNA},0}} \quad (14)$$

Eq. (14) is the predicted rate law for the cleavage of DNA catalyzed by BLM at excessive ferrous iron and oxygen concentrations. Where the Michaelis constant is

$$K_m = \frac{k_{-1}k'_{-1}}{k_1 k'_1 C_{\mathrm{Fe}^{2+},0} C_{\mathrm{O_2},0}} \quad (15)$$

Eq. (14) is in agreement with the observed rate equation (Eq. (10)). It can be seen in Table 1 that the value of K_m is approximately inversely proportional to the concentration of Fe^{2+}, therefore, supporting Eq. (15). The above analysis indicates that the proposed mechanism for the cleavage of DNA catalyzed by BLM is reasonable.

5.4. Mode of binding BLM-A_5 to DNA

As shown in Table 2, the binding of ADM to calf thymus DNA is driven entirely by a large favorable enthalpy reduction but with an unfavorable entropy decrease. In contrast, the binding of (1,10-phenanthroline)-copper to calf thymus DNA shows just an opposite thermodynamics of the reaction driven by a large favorable increase in entropy with an unfavorable raise in enthalpy. Meanwhile, the binding of BLM-A_5 to calf thymus DNA seems to be driven by a favorable entropy change with a less favorable enthalpy change. These results indicate that the thermodynamic binding behavior of BLM-A_5 ranges between those of ADM and (1,10-phenanthroline)-copper, and are in line with the suggestion made in the

references that a partial intercalation mode is involved in BLM-induced breakage of DNA [2,4–6,44,45]. The term partial intercalation used herein means that BLM inserts its bithiazole moiety, which is planar and aromatic, between adjacent base pairs of DNA, and the metal binding domain of BLM resides neatly in the minor groove of DNA [2,4–6,44,45]. The partial intercalation of BLM induces the relaxation of supercoiled DNA [4], resulting in a moderate increase in entropy.

5.5. About the self-inactivation of activated BLM

Both Fe^{2+} and O_2 serve as cofactors in DNA cleavage by BLM [2–6]. When ferrous BLM is exposed to O_2, a transient complex of drug, iron and oxygen, which is kinetically competent to initiate DNA degradation and commonly termed activated BLM, is formed [2,4,5,11,12,14,16,46].

It is indicated in this paper that the bithiazloe structure of BLM-A_5 is unchanged when it cleaves DNA as its ultraviolet spectrum is unchanged. We have thus ignored the influence of self-inactivation of activated BLM-A_5 on kinetics of the cleavage of DNA catalyzed by BLM-A_5. However, Nakamura and Peisach [47] have suggested that the bithiazloe structure of BLM-A_2 is altered when it is inactivated. It has also been shown that activated BLM-A_2 undergoes self-inactivation to a very substantial extent concomitant with its cleavage of DNA [5,46–49]. As some of the molecules become inactivated and thus are no longer capable of cleaving DNA, the measured kinetics of cleavage will lead to underestimating of the cleaving potential of the remaining molecules. It is somehow hard to understand such a large difference between the self-inactivation behavior of BLM-A_5 and BLM-A_2. In the present paper, calf thymus DNA is present when BLM-A_5 is mixed with Fe^{2+} and O_2 but not added after drug activation and it is well known that DNA does protect activated BLM against self-inactivation [5,46–49]. The validity of the thermokinetic model without considering the self-inactivation of enzyme, we proposed in this paper, has been demonstrated by a fit of the experimental data with the straight lines predicted using the model with small standard deviations. It is, therefore, speculated that the presence of DNA might fully prevent activated BLM-A_5 from self-inactivation.

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