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Thermodynamics of the cleavage of DNA induced by adriamycin: a microcalorimetric study

Yi Liang[a,b,*], Cun-Xin Wang[b], Guo-Lin Zou[c], Zhi-Yong Wang[b], Yu-Wen Liu[b], Song-Sheng Qu[b]

[a]*National Laboratory of Biomacromolecules, Institute of Biophysics, Academia Sinica, Beijing 100101, China*
[b]*College of Chemistry and Environment Science, Wuhan University, Wuhan 430072, China*
[c]*College of Life Science, Wuhan University, Wuhan 430072, China*

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Abstract

Microcalorimetry was used to measure the change in enthalpy for the scission of calf thymus DNA (ct-DNA) induced by adriamycin (ADM) in the presence of ferric ions, Vitamin C, and oxygen. At 298.15 K and pH 7.4, the overall molar reaction enthalpy for this cleavage was −147.1 kJ/mol, noticeably higher than that by the mixture of Fe^{3+}, Vitamin C, and O_2. Under the same conditions, the enthalpy change for the damage of ct-DNA by the mixture of adriamycin, ferrous ions, and oxygen, however, was nearly zero, indicating that this mixture can not induce any detectable degradation of DNA. These results suggest that both the activated adriamycin and hydroxyl radical attack DNA strands during the cleavage. A possible mechanism for the cleavage of DNA induced by adriamycin is proposed based on the calorimetric measurements. A novel thermodynamic model for the interactions of DNA with small molecules is also suggested. This is a convenient method to calculate both the binding constant (K_b) and the standard thermodynamic parameters ($\Delta_b H^0_m$, $\Delta_b G^0_m$, and $\Delta_b S^0_m$) for the binding of adriamycin-Fe^{3+} complex to ct-DNA by the calorimetric data. This nucleotide binding reaction is driven by a favorable enthalpy change, with a large unfavorable entropy change. This result indicates that the binding results in structural changes accompanied by an increase in the order of the whole system, implying that an intercalation mode is involved in adriamycin-mediated breakage of DNA.

Keywords: Adriamycin; DNA cleavage; Intercalation; Microcalorimetry; Thermodynamics

1. Introduction

The anthracycline antibiotic, adriamycin (ADM, Fig. 1), is one of the most powerful antitumor drugs in the field of cancer chemotherapy, and presently used for the clinical treatment of a broad range of human malignancies, such as leukemia and cancer of the breast, ovary, and lung [1–15]. The therapeutic effect of ADM is believed to result from its ability to induce single-strand breakage of DNA molecules in the presence of ferric ions, oxygen, and a reducing agent such as Vitamin C and sodium borohydride [2,3,5,6,9,12]. Although a significant number of experimental approaches have been used to elucidate the mechanism of the cleavage of DNA by adriamycin in the past two decades [1–15], the thermodynamic information

* Corresponding author. Tel.: +86-10-6488-8495; fax: +86-10-6487-2026.
E-mail address: liangyi@sun5.ibp.ac.cn (Y. Liang)

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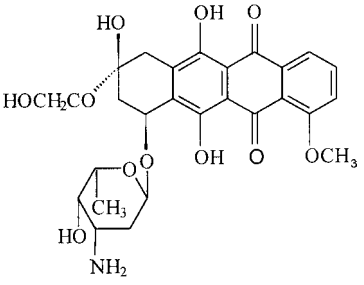

Fig. 1. Structure of adriamycin.

for the scission, which is necessary for a thorough understanding of the mechanism, has not been reported so far.

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 [16–18]. Recently, this method has yielded a large amount of useful data on the interactions between DNA and DNA-targeting molecules [11,19–27]. Among the vast amount of literature, only a limited number of authors have paid some attention to thermodynamics of the cleavage of DNA induced by DNA-targeting molecules [27].

In a previous publication from this laboratory [27], microcalorimetry was employed to check the oxidative degradation of DNA mediated by (1,10-phenanthroline)-copper(II) in the presence of 2-mercaptoethanol and oxygen, combining with the observation in agarose gel electrophoresis. In the present study, microcalorimetry was used to measure the change in enthalpy for the scission of calf thymus DNA (ct-DNA) induced by adriamycin in the presence of ferric ions, Vitamin C (Vc), and oxygen. The overall molar enthalpy change for the cleavage was reported for the first time. In order to gain insights into the nucleotide binding interactions with adriamycin, we have characterized the thermodynamic parameters for adriamycin-Fe^{3+} complex binding to ct-DNA using microcalorimetry. We were able to elucidate the binding constant (K_b) and the standard thermodynamic parameters ($\Delta_b H^0_m$, $\Delta_b G^0_m$, and $\Delta_b S^0_m$) for the binding of this complex to ct-DNA with the calorimetric data. These results help understand the binding mode of adriamycin-Fe^{3+} complex to DNA during the cleavage of DNA induced by ADM.

2. Theory and method

Understanding the thermodynamics of the binding of DNA-targeting molecules to DNA is of both practical and fundamental interests [11]. On the practical level, many DNA-targeting molecules are effective pharmaceutical agents, especially in cancer chemotherapy. On the fundamental level, DNA-targeting molecules offers a simple, well-defined system for understanding general principles that contribute to the free energy of bimolecular complex formation.

From the experiments in this laboratory, it is found that the interactions of DNA with many DNA-targeting molecules, such as adriamycin and bleomycin [28], are at rapid equilibrium:

$$\mathrm{DNA} + \mathrm{L} \rightleftharpoons \mathrm{DNA}\cdot\mathrm{L} \quad (\text{rapid equilibrium}) \tag{1}$$

where L is a DNA-targeting molecules, and DNA·L the complex between DNA and L. The intrinsic binding constant, K_b, can be defined by the following equation:

$$K_b = \frac{x}{(1-x)(C_{\mathrm{DNA},0} - nxC_{\mathrm{L},0})} \tag{2}$$

Here, $C_{\mathrm{DNA},0}$ and $C_{\mathrm{L},0}$ are the initial concentrations of DNA and L, respectively, n is the exclusion parameter which presents the number of base pairs covered by each L, and the degree of L binding to DNA, x, can be determined by the following formula:

$$x = \frac{\Delta_b H_{m,a}}{\Delta_b H^0_m} \tag{3}$$

where $\Delta_b H^0_m$ is the standard binding enthalpy per mole of L, and $\Delta_b H_{m,a}$ the apparent molar binding enthalpy which can be calculated by

$$\Delta_b H_{m,a} = \frac{Q_{2,\infty}}{C_{\mathrm{L},0}\cdot V_T} \tag{4}$$

from the calorimetric data. Here, $Q_{2,\infty}$ is the total heat effect of L binding to DNA, and V_T the total volume of the reacting system (in this paper, V_T=6.00 cm^3)

Writing

$$r = \frac{n_{\mathrm{DNA},0}}{n_{\mathrm{L},0}} = \frac{C_{\mathrm{DNA},0}}{C_{\mathrm{L},0}} \tag{5}$$

where r is the molar ratio of DNA to L.

Substituting Eqs. (3) and (5) into Eq. (2), we get

$$r = \frac{C_{\mathrm{DNA},0} K_\mathrm{b} n \Delta_\mathrm{b} H_{\mathrm{m,a}} (\Delta_\mathrm{b} H^0_\mathrm{m} - \Delta_\mathrm{b} H_{\mathrm{m,a}})}{C_{\mathrm{DNA},0} K_\mathrm{b} (\Delta_\mathrm{b} H^0_\mathrm{m})^2 - (C_{\mathrm{DNA},0} K_\mathrm{b} + 1) \Delta_\mathrm{b} H_{\mathrm{m,a}} \Delta_\mathrm{b} H^0_\mathrm{m}} \quad (6)$$

This novel thermodynamic model can be used to perform a non-linear least-squares analysis of the molar ratio r as an explicit function of the apparent molar binding enthalpy $\Delta_\mathrm{b} H_{\mathrm{m,a}}$. Then three unknown binding parameters, K_b, $\Delta_\mathrm{b} H^0_\mathrm{m}$, and n, can be obtained by fitting the experimental data (r and $\Delta_\mathrm{b} H_{\mathrm{m,a}}$) to the model using the Origin software provided by Microcal Software, Inc. (Version 5.0). Also the appropriateness of the model can be tested statistically (e.g., using χ^2 test).

From the binding constant (K_b) and the standard molar binding enthalpy ($\Delta_\mathrm{b} H^0_\mathrm{m}$), the standard molar binding free energy ($\Delta_\mathrm{b} G^0_\mathrm{m}$) and the standard molar binding entropy ($\Delta_\mathrm{b} S^0_\mathrm{m}$) for the binding reaction can be calculated by the following relations, respectively:

$$\Delta_\mathrm{b} G^0_\mathrm{m} = -RT \ln K_\mathrm{b} \quad (7)$$

$$\Delta_\mathrm{b} S^0_\mathrm{m} = \frac{\Delta_\mathrm{b} H^0_\mathrm{m} - \Delta_\mathrm{b} G^0_\mathrm{m}}{T} \quad (8)$$

3. Experimental

3.1. Reaction systems

Four reaction systems, as shown below, were studied in sequence, and the cleavage and binding reactions were undertaken in 0.1 mol dm^{-3} Tris–HCl buffer (pH 7.4) at 25.00 and 37.00°C, respectively.

- Reaction 1: the cleavage of calf thymus DNA induced by adriamycin in the presence of ferric ions, Vitamin C, and oxygen.
- Reaction 2: the cleavage of calf thymus DNA by the mixture of ferric ions, Vitamin C, and oxygen.
- Reaction 3: the cleavage of calf thymus DNA by the mixture of adriamycin, ferrous ions, and oxygen.
- Reaction 4: the binding of adriamycin-Fe^{3+} complex to calf thymus DNA.

3.2. Reagents

Calf thymus DNA (Sigma) 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 the purified DNA were measured at room temperature. From the results, the ratio of A_{260} to A_{280} is over 1.8. Adriamycin was the product of Haimen Pharmaceutical Factory, Zhejiang, China, and a molar extinction coefficient of 11500 dm^3 mol^{-1} cm^{-1} at 480 nm was used for free adriamycin. $FeCl_2 \cdot 4H_2O$ (A.R. grade) were purchased from Merck's reagent, Germany. Other chemicals used were domestic and of A.R. grade. All reagent solutions were prepared in 0.1 mol dm^{-3} Tris–HCl buffer (pH=7.4). Because both the $FeCl_2$ and Vitamin C solutions were easily oxidized by oxygen, they were placed in brown bottles and then flushed with purified nitrogen for 10 min, sealed, and stored in a refrigerator prior to use. Moreover, they were freshly prepared on each occasion.

3.3. Instrumentation

The heats of the reactions mentioned above were determined at pH 7.4 by using a LKB-2107 batch microcalorimeter system from Sweden. The instrument contains twin calorimeter cells, one of which is the reaction cell and the other a reference cell, each cell being divided into two parts [27–31]. Compartment I of reaction cell contained 2.00 cm^3 of Vitamin C, $FeCl_2$, and DNA solutions for reactions 1/2, 3, and 4, respectively, and compartment II of reaction cell contained 4.00 cm^3 of DNA-ADM-$FeCl_3$-O_2, DNA-$FeCl_3$-O_2, DNA-ADM-O_2 mixtures, and adriamycin-Fe^{3+} complex for reactions 1, 2, 3, and 4, respectively. During the addition of samples, the oxygen in air may be dissolved in the $FeCl_2$ and Vitamin C solutions again, and therefore the operation must be performed carefully and rapidly. Before samples were added, a clean, long, and soft tubing was inserted in the reaction cell and purified N_2 introduced into the cell to purge the oxygen in the cell. When samples were added, the pipe was moved to the mouth of the reaction cell and purified N_2 continuously introduced into the cell. Owing to communication of the Compartments I and II, samples could be added to I at first while purified N_2 might be introduced into II, and then these

two operations were exchanged. After samples had been added, the source of N_2 was removed, and the plug for the reaction cell was closed tightly at once. The same operational procedure was adopted for adding samples to the reference cell. In order to avoid the influence of the heat effects of diluting and mixing etc. on the results of the measurement, the contents and quantities in both cells were as close as possible except that DNA was not added to the reference cell.

4. Results

4.1. Enthalpy of the cleavage of DNA induced by adriamycin

Fig. 2 shows an experimental calorimetric curve for the cleavage of calf thymus DNA induced by adriamycin in the presence of ferric ions, Vitamin C, and oxygen at 298.15 K and pH 7.4. The calorimetric curve for this DNA cleavage shows a flat exothermic peak, indicating the change in enthalpy for the cleavage was negative under the conditions used. The overall molar reaction enthalpy for Reaction 1 in the presence of excessive ferric ions, Vitamin C, and oxygen, $\Delta_r H_m$, can be calculated by

$$\Delta_r H_m = \frac{Q_\infty}{V_T \cdot C_{DNA,0}} \quad (9)$$

Here, Q_∞ is the total heat effect of Reaction 1, and $C_{DNA,0}$ is the initial concentration of ct-DNA. The calculated results are listed in Table 1. As can be seen from Table 1 and Fig. 2, this DNA cleavage is a largely exothermic and enthalpy-driven reaction.

In order to gain insights into the mechanism of adriamycin-induced cleavage of DNA, we have used microcalorimetry to measure the overall changes in enthalpy for two control reactions, namely the scission of ct-DNA by the mixture of Fe^{3+}, Vitamin C, and O_2 and the mixture of adriamycin, Fe^{2+}, and O_2. Fig. 2 also displays two calorimetric curves for these reactions at 298.15 K and pH 7.4. The calculated results are summarized in Table 2.

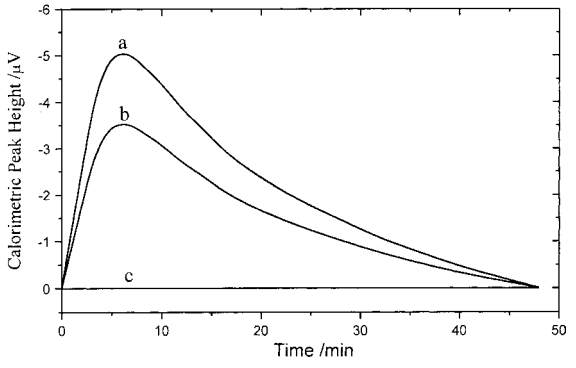

Fig. 2. Calorimetric curves for the scission of calf thymus DNA by (a) the mixture of adriamycin, Fe^{3+}, Vitamin C, and O_2; (b) the mixture of Fe^{3+}, Vitamin C, and O_2; (c) the mixture of adriamycin, Fe^{2+}, and O_2. The experimental conditions were T=298.15 K, pH=7. (a) $C_{DNA,0}=2.565\times10^{-4}$ mol dm^{-3}, $C_{ADM,0}=5.750\times10^{-6}$ mol dm^{-3}, $C_{FeCl_3,0}=3.380\times10^{-4}$ mol dm^{-3}, $C_{Vc,0}=6.450\times10^{-4}$ mol dm^{-3}, and oxygen was in excess. (b) $C_{ADM,0}=0$, and the other conditions are the same as those in (a). (c) $C_{DNA,0}=1.364\times10^{-4}$ mol dm^{-3}, $C_{ADM,0}=5.750\times10^{-5}$ mol dm^{-3}, $C_{FeCl_2,0}=3.380\times10^{-4}$ mol dm^{-3}, and oxygen was in excess.

Table 1
Initial concentrations of DNA and O_2, total heat effect (Q_∞), and molar enthalpy change for the scission of calf thymus DNA induced by adriamycin in the presence of Fe^{3+}, Vitamin C, and O_2 at 298.15 K and pH 7.4[a]

$C_{DNA,0}\times10^4$ (mol dm^{-3})	$C_{O_2,0}\times10^4$ (mol dm^{-3})	$-Q_\infty$ (mJ)	$-\Delta_r H_m$ (kJ mol^{-1})
0.6413	6.15	59.46	154.5
1.283	5.98	113.8	147.9
2.565	5.65	229.0	148.8
2.565	5.65	225.3	146.4
3.848	5.32	317.9	137.7
Average value			147.1±6.1

[a] $C_{ADM,0}=5.750\times10^{-6}$ mol dm^{-3}, $C_{FeCl_3,0}=3.380\times10^{-4}$ mol dm^{-3}, and $C_{Vc,0}=6.450\times10^{-4}$ mol dm^{-3}.

Table 2
Molar enthalpy changes for the cleavages of calf thymus DNA by the mixture of adriamycin, Fe^{3+}, Vitamin C, and O_2, by the mixture of Fe^{3+}, Vitamin C, and O_2, and by the mixture of adriamycin, Fe^{2+}, and O_2 at 298.15 K and pH 7.4

Cleavage systems	$\Delta_r H_m$ (kJ mol^{-1})	Products
ADM-Fe^{3+}-Vc-O_2	−147.1±6.1	DNA fragments [12]
Fe^{3+}-Vc-O_2	−102.2±4.3	DNA fragments [9,32]
ADM-Fe^{2+}-O_2	0	No detectable degradation of DNA

Table 3
Thermodynamic parameters for the binding of adriamycin-Fe^{3+} complex to calf thymus DNA at 310.15 K and pH 7.4[a]

$K_b \times 10^{-5}$	n	$\Delta_b H_m^0$(kJ mol^{-1})	$\Delta_b G_m^0$(kJ mol^{-1})	$\Delta_b S_m^0$(J mol^{-1} K^{-1})	Action mode
1.32±0.10	4.96±0.59	−41.6±0.4	−30.4±0.2	−36.1±1.9	Intercalation

[a] For experimental conditions, see Fig. 3.

4.2. Thermodynamic parameters for the binding of adriamycin-Fe^{3+} complex to DNA

In order to gain insights into the nucleotide binding interactions with adriamycin, we have characterized the thermodynamic parameters for the binding of adriamycin-Fe^{3+} complex to ct-DNA by using microcalorimetry.

Table 3 summarizes the thermodynamic data for this binding reaction at 310.15 K and pH 7.4, in which the values of K_b, $\Delta_b H_m^0$, and n are obtained by fitting the experimental data (the hollow circles in Fig. 3) to Eq. (6). Then the remaining standard thermodynamic parameters for this binding, $\Delta_b G_m^0$ and $\Delta_b S_m^0$, are calculated by Eqs. (7) and (8), respectively. The solid line in Fig. 3 is the apparent molar enthalpy change for this binding as predicted by the parameters in Table 3. The standard relative errors on these parameters are small, indicating that the thermodynamic model for the binding of DNA-targeting molecules to DNA proposed in this paper, is reasonable.

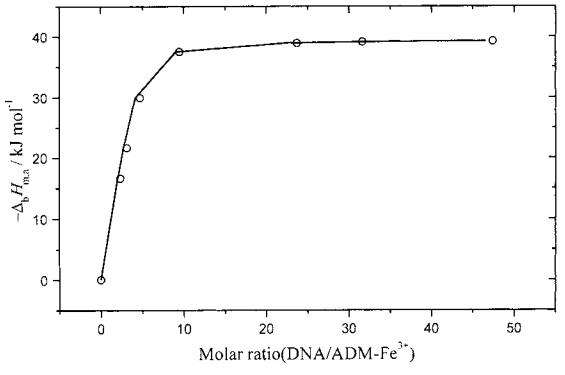

Fig. 3. Plot of the apparent molar enthalpy change for the binding of adriamycin-Fe^{3+} complex to calf thymus DNA against the molar ratio of DNA to the complex. The experimental conditions were T=310.15 K, pH=7.4, and the initial concentration of calf thymus DNA was 1.364×10^{-4} mol dm^{-3}. The hollow circles are the experimental data, and the solid line is the theoretical curve predicted by the thermodynamic model (Eq. (6)) and the parameters in Table 3.

5. Discussion

5.1. Mechanism for the cleavage of DNA induced by adriamycin

From electrophoresis experiments on agarose gel, it was found that the mixture of adriamycin, Fe^{3+}, Vitamin C, and O_2 as well as the mixture of Fe^{3+}, Vitamin C, and O_2 nicked pBR-322 DNA and λ DNA to small DNA fragments [9,12,32]. It was also demonstrated that the latter mixture did damage DNA by way of hydroxyl radical [9,32]. As we can see from Table 2, under the conditions presented here, the overall molar reaction enthalpy for the cleavage of DNA induced by the mixture of adriamycin, Fe^{3+}, Vitamin C, and O_2 was −147.1 kJ/mol, noticeably higher than that by the mixture of Fe^{3+}, Vitamin C, and O_2. This suggests that the former mixture can attack DNA strands through at least two paths. One is the way of hydroxyl radical where ADM is not necessary. The other is the way relating to adriamycin, in which ADM is necessary. Someya and co-workers have assumed that this DNA cleavage is induced by attack of the free radical of ADM, which is produced by the auto-oxidation of the quinone moiety of ADM [3]. In other words, they suggested that the second path should be the way of free radical of ADM. However, as shown in Table 2, the enthalpy change for the damage of ct-DNA by the mixture of adriamycin, ferrous ions, and oxygen without adding hydrogen peroxide was nearly zero, clearly indicating that this mixture can not induce any detectable degradation of DNA. This result suggests that it is the activated adriamycin rather than free radical of adriamycin that is involved in ADM-mediated breakage of DNA. Therefore, we conclude that both the activated adria-

mycin and hydroxyl radical attack DNA strands during the cleavage.

According to the above experimental results, we proposed a possible mechanism for the cleavage of DNA induced by adriamycin in the presence of Fe^{3+}, Vitamin C, and O_2. For the attack due to the hydroxyl radical:

$$Fe^{3+} + Vc + O_2 \rightarrow Fe^{2+} + \text{Dehydroascorbic acid} + H_2O_2 \quad [33] \quad (10)$$

$$Fe^{2+} + H_2O_2 \rightarrow {}^{\bullet}OH + OH^- + Fe^{3+} \quad [9] \quad (11)$$

$$DNA + {}^{\bullet}OH \rightarrow \text{DNA fragments} \quad [3] \quad (12)$$

For the attack due to the activated adriamycin:

$$ADM + Fe^{2+} + H_2O_2 + O_2 \rightleftharpoons \text{Activated ADM} \quad (13)$$

$$DNA + \text{Activated ADM} \rightleftharpoons DNA \cdot \text{Activated ADM} \rightarrow \text{DNA fragments} + ADM + Fe^{3+} + O_2 \quad (14)$$

5.2. Mode of binding adriamycin-Fe^{3+} complex to DNA

Double-helical DNA can bind different types of DNA-targeting molecules which can be classified in two categories: intercalators which insert their aromatic ring between adjacent base pairs, and groove binders which bind DNA within either groove of the double helix [34].

As we can see from Table 3, the binding of adriamycin-Fe^{3+} complex to calf thymus DNA is driven by a favorable enthalpy change, with a large unfavorable entropy change. This result indicates that the binding results in structural changes accompanied by an increase in the order of the whole system, implying that an intercalation mode is involved in adriamycin-mediated breakage of DNA.

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