INTERACTION OF DOXORUBICIN WITH DNA-HMG1 COMPLEX

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Abstract

In this study, the interaction of the anthracycline antibiotic doxorubicin with DNA-HMG1 complex was investigated employing UV/VIS spectroscopy, thermal denaturation and DNA cellulose chromatography techniques. The results indicated that the binding of doxorubicin to the protein reduces its Tm in a dose dependent manner. Although doxorubicin protects free DNA against thermal denaturation in the presence of HMG1, thermal melts represent biphasic profile corresponding to DNA- drug, HMG1- drug and free components. Interaction of antibiotic with DNA - HMG1 complex using DNA cellulose columns also implies that the drug binds strongly to single-stranded DNA-HMG1 complex rather than to double- stranded DNA-HMG1 columns. The results suggest destabilization of HMG1 protein by drug action thereby preventing DNA-HMG1 complex formation.

Introduction

The anthracycline antibiotic doxorubicin (adriamycin) is an effective antitumor agent widely used in the treatment of both leukemia and solid tumors [1]. The mechanism of action of doxorubicin is generally considered to involve intercalation into DNA double helix and inhibition of DNA and RNA synthesis [2,3]. In the cell nucleus, however, DNA is not naked but is complexed with special sets of chromosomal proteins named histones and nonhistone proteins [4].

High mobility group (HMG) proteins are a family of nonhistone protein components of relatively low molecular weights in chromatin [4,5]. These proteins are widely distributed in eukaryotic cells and constitute several fractions mainly HMG 1, 2, 14 and 17 [6,8]. HMG1, which is the subject of this study, is a protein rich in both basic and acidic amino acids. It shows preferential binding to single-stranded DNA [9] and unwinds double-stranded DNA structure [10, 11]. Although the *in vivo* function of this protein has not yet been shown, there are some indications of its involvement in DNA replication or RNA transcription [12,13].

The interaction of doxorubicin with crude chromatin and nucleosomes has been the subject of some reports [14,15]. Previously, we also examined the binding of doxorubicin to DNA-histone complexes in solution and showed that histones also contribute to the DNA-drug interactions [16]. In this study, we investigated the effect of doxorubicin on DNA-HMG1 chromatin, in order to elucidate the possible influence of this protein on DNA-drug interaction processes.

Keywords: HMG proteins; Doxorubicin; Nonhistone proteins

Experimental Section

Protein Preparation

Total HMG proteins were isolated from calf thymus either using 0.35 M NaCl extraction ammonium sulfate solubilization procedure followed by dialysis and freeze-drying [17] or by perchloric acid (PCA) extraction and acetone precipitation method as described previously [8]. Approximately 0.5 g of the protein was fractionated on Carboxymethyl Sephadex - C25 column (30 × 350 mm) previously equilibrated with 7.5 mM borate buffer pH 9

[6]. HMG1 was eluted with a pregradient wash and collected by dialysis via several changes of 50 mM tris-HCl buffer and then freeze-dried or acetone-precipitated. The protein was further purified by recycling through a CM-Sephadex column (1.5×15 cm). Purity of HMG1 was determined using SDS polyacrylamide gel electrophoresis described by Laemmli [18] with some modifications as follows: 15% acrylamide in separating gel; 4% acrylamide in stacking and 1.8% glycine in the electrode buffer.

Stock Solutions

HMG1 at a concentration of 4 mg/ml was prepared in 50 mM tris- HCl buffer pH 8 divided into several portions and frozen at - 20°C until use. No turbidity, degradation or any changes in its optical density occurred for at least three months. The amount of HMG1 in the solution was either measured by Lowry method modified by Hartree [19] using bovine serum albumin as a standard or by recording UV spectra and using the published extinction coefficient of 20,900 at 280 nm [20]. Sodium chloride was freshly dissolved in tris buffer and added to the reaction mixtures immediately before incubation.

Doxorubicin (or adriablastin) was purchased from Sigma chemical company and used without further purification. It was dissolved in distilled water at a final concentration of 1 mg/ ml and stored in a dark bottle at 4°C for not more than a week prior to use. For longer periods of time, drug samples were stored at -20°C.

Calf thymus DNA was from Sigma or prepared according to the method of Kay *et al.* [21]. It was dissolved in 50 mM tris buffer pH 8 at a concentration of 1 mg/ml by stirring overnight at 4°C. The sample was then dialysed for 15 hours against three changes of buffer and the accurate concentration was determined optically using E260 of 6800 as described in [22]. The DNA molecule showed about 5148 base pairs when its size was measured on 5% TBE (tris-borate - EDTA) polyacrylamide gel electrophoresis aligned with DNA marker [23].

Binding Measurements

HMG1 protein (0.1 mg/ml) was mixed with different concentrations of doxorubicin (0.1 - 70 μg/ml) in 50 mM tris buffer pH 8 and the volumes were adjusted to 1 ml. The pH of all samples were checked before incubation and if required they were adjusted to pH 8. Treated and control samples were then vortexed and incubated at 23-25°C for 30 min in the dark while occasionally shaking.

The interaction of doxorubicin with DNA-HMG1 complex was performed at a DNA to protein ratio of 1:2. A stock solution of DNA, prepared as explained above, was diluted to 1:20 with the same buffer to give an absorbance of 0.6-0.8 at 260 nm (this is equal to 0.05 mgDNA/ml). The antibiotic was then added to the mixture and incubated at 25°C. The final volume of each sample was 1 ml. DNA, HMG1 and the drug were mixed together simultaneously or in 30 min intervals, that is DNA was first interacted with HMG1 for 30 min, then drug solutions were added and the incubation was continued for another 30 min under the same conditions.

Thermal Analysis

Doxorubicin treated and the controls were subjected to an increase in temperature at a rate of 1°C/min. The absorbances were read at 260 nm on a single beam Gilford 240 spectrophotometer using tris buffer as a blank. In some cases the absorbance at 280 nm was also monitored. Derivative of hyperchromicity at temperature T was calculated using the following equation

$$\frac{dh(T)}{dT} = \frac{h(T+1) - h(T-1)}{2}$$

in which dh is derivative of hyperchromicity and h is hyperchromicity [24].

Difference Spectra

Mixtures of doxorubicin and DNA- HMG1 in 50 mM tris - HCl buffer pH 8 and buffer containing 0.1 M NaCl were prepared as described above, and after incubation, difference spectra were drawn by scanning the samples against the buffer or the same concentration of the drug. Spectra were scanned between 190-350 and 300-700 nm using a Shimadzu UV-260 model spectrophotometer.

DNA - Cellulose Chromatography

Single-stranded DNA cellulose was purchased from Sigma chemical company. Double-stranded DNA-cellulose was prepared according to the method of Alberts *et al.* [25] using 1 mg DNA per gram of çellulose.

After several washes of DNA- cellulose mixtures with the column buffer (10 mM tris-HCl, 1 mM EDTA pH 8.0) containing 0.05 M NaCl, the slurries were packed into pasteur pipets (0.5 × 1.5 cm) plugged with glass wool. Column washings were continued with the same buffer at a flow rate of 3 ml/hour. For DNA-drug interactions, in a series of experiments, doxorubicin at a concentration of 50 μg/ml was loaded onto the columns. Single-, and double-stranded columns were made in the same size and condition.

Elutions were carried out by buffer, 0.05 - 1 M NaCl salt gradient and followed by 5 M urea. 3 ml fractions were collected and the optical densities were measured at 480 and 260 nm. In the same way, HMG1 was first interacted with DNA and after washing out the unbound protein from the column, doxorubicin was loaded onto the column and elution was continued as described above. Per cent of

binding affinity was estimated by calculating the area under the peaks.

Results

Thermal Analysis

Purity of the protein was first determined by SDS gel electrophoresis which showed a single band at HMG1 position when run parallel to total calf thymus HMG proteins. All experiments were carried out at pH 8 to avoid protein aggregation and precipitation at its isoelectric point. Absorbance measurements were carried out at 260 nm in order to compare the results with those of HMG1 - DNA complexes (see below).

In order to study the interaction of doxorubicin with DNA - HMG1 complex, it was necessary, initially, to examine the possible effect of the drug on free DNA and HMG1. For this propose, DNA at a concentration of 0.05 mg/ml was incubated with various concentrations of the drug and then the samples were subjected to Tm measurements. Thermal denaturation profiles in both 50 mM tris buffer pH 8 (a and b) and in 0.1 M salt condition (c and d) are compared in Figure 1. DNA, with an approximate base pair of 5148, shows Tm of 75°C and 80°C in the presence and absence of salt respectively. As is seen, the interaction of doxorubicin with DNA stabilizes it against thermal melting since Tm is increased from 75°C to 89°C when 30 µg/ml of the drug is used (Δ T is 14°C). The process is also ionic strength dependent.

The interaction of various concentrations of doxorubicin (0.1-70 µg/ml) progressively reduces its Tm reflecting values of 53, 48, 43 and 38°C respectively. Destabilization effect of the antibiotic is expressed as a difference between the Tm of complexes and that of free HMG1 (in the absence of the drug; ΔT). Therefore, 17°C transition in Tm value is observed when 70 µg/ml of doxorubicin is considered. The ionic strength dependency of the melting profiles in the presence and absence of the drug was studied in the same buffer containing 0.05 M and 0.1 M sodium chloride. The profile given in Figure 2 shows Tm transition of at least seven degrees to the higher temperatures.

HMG1 is a DNA binding protein in which its interaction with both single- and double-stranded DNA has been reported [9,10]. In agreement with the previously reported results [11], our data obtained from Tm measurements of DNA-HMG1 complex (Fig. 3) indicate that the binding of HMG1 to DNA raises Tm of DNA from 75°C to 81°C in tris buffer and at higher ionic strengths (e.g. presence of 0.1 M NaCl in the incubation medium) a biphasic profile is observed suggesting unwinding of DNA molecule by HMG1 action (in all experiments a DNA to protein ratio of 1:2 was used). When HMG1 and doxorubicin were added simultaneously to DNA solutions and incubated as

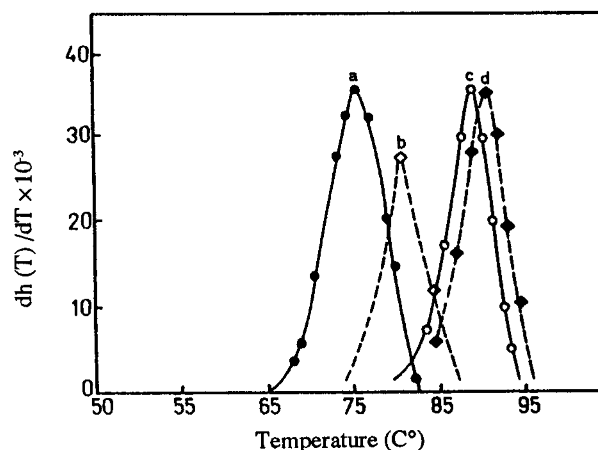

Figure 1. Derivative thermal denaturation profiles of DNA in buffer (a) and salt condition (b). DNA-drug complex in tris buffer (c) and in buffer plus 0.1 M salt (d).

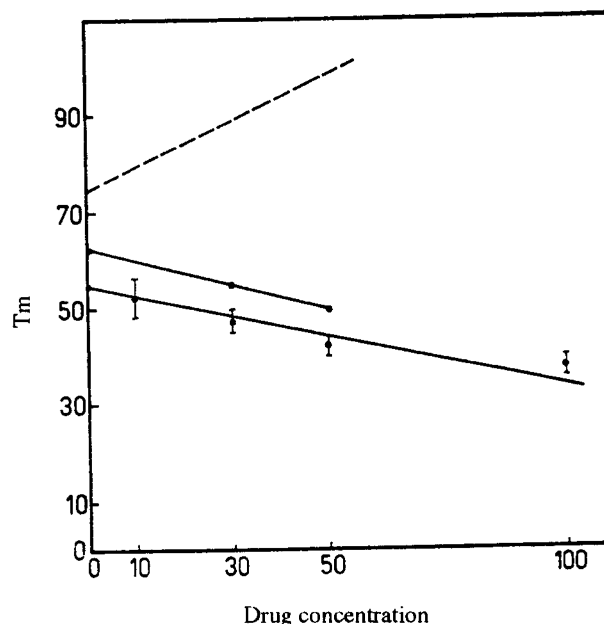

Figure 2. Relationship between the Tm changes as a function of drug concentration ●–●; HMG1-drug in tris buffer ○–○; HMG1-drug in 0.1 M NaCl and - - -; DNA-drug in buffer.

described in the method section, two Tm peaks appeared (Fig. 3a), one at 89°C and the other at 47°C. These Tm values seem to correspond to DNA-drug and HMG1-drug complexes respectively. Figure 3b represents thermal denaturation profiles obtained from the interaction of three components, DNA, HMG1 and the drug, with each other in the salt condition and show three peaks at temperatures 93°C, 62°C and 56°C. Comparing the results with those above indicate that the Tm values correspond to DNA-

drug, free HMG1 and HMG1-drug complexes respectively (from right to left).

Spectroscopy

UV absorption and difference spectra were recorded at wavelengths between 190-700 nm. The results of 30 µg/ml doxorubicin treatments are given in Figure 4. Any change in the absorbance at 480 nm (specific absorbance region of the drug) can give useful information about drug action in the mixture.

When the spectra are recorded between 350-700 nm (Fig. 4a) a considerable change is observed. Absorbance reduction at 480 nm implies drug binding to the macromolecules of the mixture. It is also seen that alterations in the spectra pattern between 190-350 nm (Fig. 4b) are more sensible compared to 480 nm pattern, thus, apart from hypochromicity, two special peaks appear on the spectrum at 244 and 264 nm. These spectral shifts to higher wavelengths are presumably related to the structural changes in the protein, DNA or DNA-protein complex brought about by drug binding. Drawing difference spectra of DNA-HMG1 complex against DNA or HMG1 (results not shown) indicate that reduction of absorbance at 210-230 nm corresponds to DNA-HMG1 complex formation but changes in the regions of 230-300 nm are produced by drug action.

DNA-Cellulose Binding Assay

For further elucidation of the influence of the drug on DNA-HMG1 complex, an attempt was made to measure the extent and affinity of the binding using DNA-cellulose columns. For this propose, first the binding of doxorubicin to single- and double-stranded DNA (ss-DNA and ds-DNA) was designed. Two columns were set up with identical conditions, one with ss-DNA and the other with ds-DNA. After drug loading (50 µg/column), the columns were washed with several volumes of tris buffer containing 0.05 M NaCl to remove unbound or excess drug from the columns (red colour of doxorubicin, apparently, shows its distribution between the solid and mobile phases). Then a salt gradient of 0.05 -1 M was applied followed by elution with urea in buffer.

The results obtained from the absorbance measurements at 480 nm are shown in Figure 5a. As the chromatograms show, doxorubicin binds to both ss- and ds-DNA but the extent of its affinity to ss-DNA is remarkably higher. In the ss-DNA-cellulose column, a small amount of the drug (10-15%) elutes with low concentrations of salt (0.05 M NaCl), approximately 30% elutes after applying urea and at the end still nearly 60% of the drug remains attached to DNA inside the column indicating that a strong interaction has been produced between doxorubicin and DNA molecules. On the other hand, in the double-stranded DNA column nearly 25% of the drug is released as elution begins, and 30-35% in the presence of higher quantities of salt and

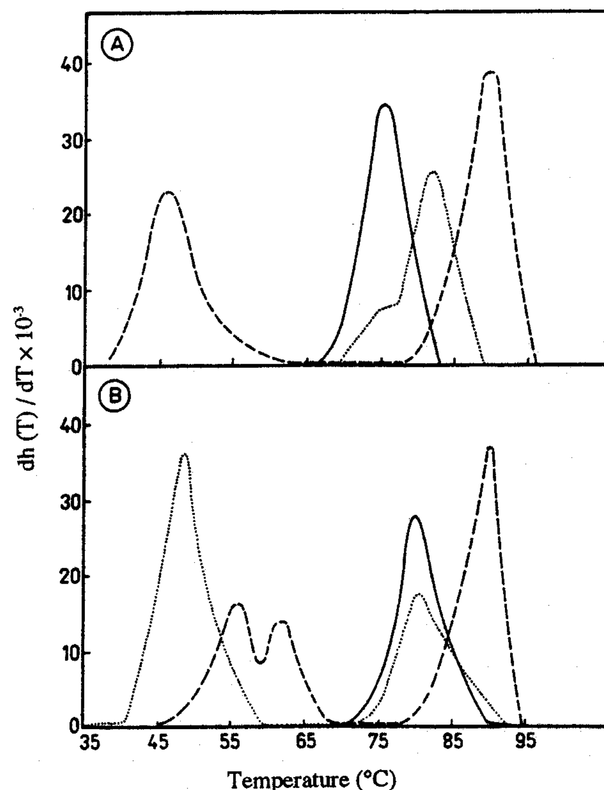

Figure 3. Melting profiles obtained from the interaction of doxorubicin with DNA-HMG1. a: Profiles in tris buffer pH 8 and b: profiles in salt condition. The results are means of five separate experiments. ; DNA, ...; DNA-HMG1, - - - ; interaction of drug with DNA-HMG1 complex. DNA/protein ratio of 1:2 and drug concentration of 30 µg/ml is presented.

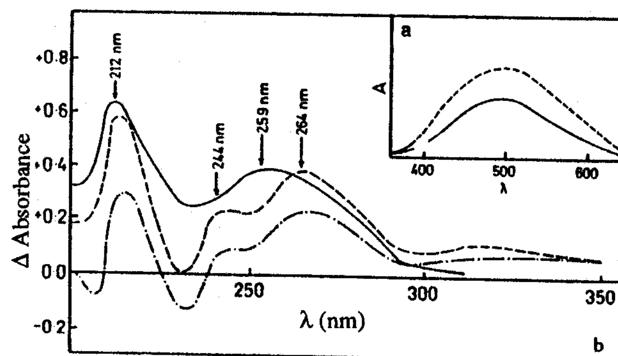

Figure 4. a: Absorption spectra of - - - ; 30 µg/ml doxorubicin, ; DNA-HMG1-drug in buffer.
b: Difference spectra between 190-350 nm of ; DNA-HMG1 against buffer, - - - ; and —·—·— ; are DNA-HMG1- drug against the same amount of drug in buffer and salt conditions respectively.

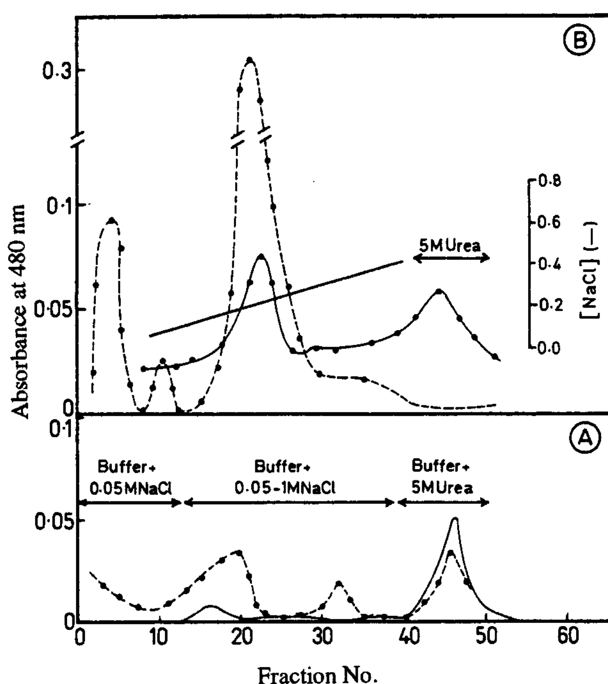

Figure 5. Interaction of doxorubicin with the ss-DNA- cellulose ( ) and ds-DNA- cellulose (—·—·—) columns. 50 μg/ml of drug was loaded onto 1 ml columns containing 1 mg DNA/ml.
b: The effect of the drug on DNA-HMG1 complex prepared in the ss- and ds-DNA-cellulose columns. ●—●; ss- DNA-HMG1 and ●- - -●; ds-DNA-HMG1 columns. The salt gradient of 0.05-1 M NaCl and then urea in buffer were used for elution. Results are the means of four experiments.

urea. Therefore, at the end, the amount of drug remaining inside the column is 40% showing a lower affinity of drug to ds-DNA compared to the ss-DNA under the experimental conditions used.

In the next experiment, HMG1 was first bound to ss- or ds- DNA cellulose column and then the binding behaviour of antibiotic to the DNA- HMG1 complex was examined. The chromatograms thus obtained are given in Figure 5b. It is shown that in ds-DNA column nearly 30% of the drug comes out with the washing buffer and the rest with 0.15 M NaCl solution. In this case, the amount of drug left inside the column is negligible (5%) suggesting that the affinity of doxorubicin to DNA-HMG1 complex is very weak. However, on the column containing ss-DNA-HMG1, a small amount of doxorubicin (25%) elutes at 0.2 M NaCl concentration, the same amount with 5 M urea and at the end still 50% of the drug remains bound to the column, producing a red-coloured resin.

Discussion

The biological activity of a chemotherapeutic agent is related to its physicochemical interaction with biological receptors. The activity of anthracycline antibiotic, doxorubicin, as an anticancer agent is due mainly to its specific DNA damaging properties which interfere with DNA replication and transcription processes and probably lead to the therapeutic effect [2,3]. The mode of doxorubicin action has become the subject of many reports showing that it intercalates into DNA double-strand [2,3] and also produces single- and double-strand breaks in DNA, therefore DNA has been introduced as a main target for drugs action [28, 29]. In addition, several recent studies have examined the effects of the drug at the membrane level [30, 31].

Our results suggest that in the cell, apart from targets mentioned for doxorubicin, chromosomal proteins should be taken into consideration. A preliminary investigation carried out in our laboratory into the interaction of doxorubicin with the DNA-histone complexes in solution has been published [16] and there are also some reports showing the effect of this drug on isolated chromatin [14] and nucleosomes [15, 32]. The present study demonstrates that doxorubicin interacts with the nonhistone chromosomal protein HMG1 and reduces its melting temperature. Destabilization of the protein by drug action seems to be highly strong and probably proceeds by a structural or conformational change of protein.

The results also demonstrate that doxorubicin binds to DNA but the effect is completely different from HMG1-drug complex. Indeed, DNA structure is stabilized by drug binding and its Tm shifts to higher temperatures. This has also been reported for the interaction of actinomycin D, daunomycin and nogalomycin with DNA [2, 33, 34].

HMG1 is a low molecular weight DNA binding protein known as a potential candidate for affecting structural features of DNA. These structural changes almost certainly play a key role in the regulation of gene expression. It has been shown that the interaction of HMG1 with DNA is ionic strength dependent, in the presence of salt HMG1 proceeds unwinding of circular or linear DNA [10, 37] but under conditions of no added salt, it stabilizes DNA against thermal denaturation [38,39]. The results presented here not only support the above findings but also demonstrate that when the interactions are performed in the presence of doxorubicin, Tm changes belong to direct interaction of doxorubicin with DNA or HMG1 individually. It is obviously clear that the drug competes with HMG1 in binding to DNA and in this sense, prevents DNA-HMG1 complex formation or the binding of the drug to DNA compacts it in such a way that HMG1 will not be able to interact with it. Moreover, the other possibility is that the binding of the drug to HMG1 alters the protein structure in such a way that reduces its binding affinity to DNA. The same observations are found for the experiments carried out on DNA-cellulose columns in the presence and

absence of HMG1. The results are coincident with those presented in the Tm and spectra section.

Assuming that HMG1 plays a role in genome function; these preliminary results imply that doxorubicin, as an antitumor agent, not only binds to DNA chain in cellular chromatin but presumably interacts with the chromosomal proteins and thereby affects DNA-protein contacts. It is recognized of course that the *in vivo* situation is more complex, therefore other factors should also be taken into consideration. Work is now in progress to invest igate the interaction of anthracycline antibiotics with other nuclear proteins essentially in reconstituted and native chromatin to obtain more information about the mechanism of drug action at the molecular level.

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