The differential effects produced by *cis*- and *trans*-DDP on DNA in calf thymus nucleosomes

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Calf thymus nucleosomes containing H1 were treated with dichlorodiammineplatinum (DDP) at low binding ratios ($r = 0.05–0.15$). Change in the electrophoretic mobility of the extracted nucleosomal DNA was observed following treatment with *cis*-DDP and little change with *trans*-DDP. There was a decrease in the electrophoretic mobility of the nucleosomal DNA as well as obliteration of the nucleosomal repeat distance. The fluorescence intensity of terbium binding to the extracted DNA showed minimal change following drug treatment. However, the thermal melting behavior of the nucleosomal DNA was altered to a greater extent following *cis*-DDP treatment at 280 rather than 260 nm and a destabilization of the DNA helix was observed. These data suggest that in the whole nucleosome, *cis*-DDP produces greater structural effects on the packaged DNA than *trans*-DDP, although similar amounts of drug are bound with both isomers.

cis-*DDP* *Nucleosome* *DNA melting profile*

1. INTRODUCTION

cis-Dichlorodiammineplatinum (*cis*-DDP) is a potent anti-tumor drug now in use clinically to treat head, neck and testicular cancer and recently lymphoma. The *trans* isomer possesses little to no anti-tumor activity. DNA is thought to be the prime target for *cis*-DDP [1,2]. The ensuing lesion which presumably inhibits DNA synthesis is thought to be either interstrand [3,4] or intrastrand crosslinks [5]. However, the DNA in eukaryotic cells is associated with histone and non-histone proteins and, thus, the possibility of DNA-protein crosslinks must also be considered [6]. *trans*-DDP has also been shown to produce crosslinks, although this isomer is thought to be more effective in producing protein-protein and protein-DNA crosslinks than *cis*-DDP [7]. The *trans* isomer does produce DNA crosslinks as well, but much more slowly and at higher drug concentrations than does *cis*-DDP. Limited reports have appeared studying the effect of these drugs on chromatin or nucleosomes. The first such investigation in [7] employed nucleosome core particles and showed the formation of DNA-protein as well as protein-protein crosslinks. We have employed fluorescent probes [8], and showed that at very low drug concentrations both *cis*- and *trans*-DDP produce effects on the histone proteins in calf thymus nucleosomes. We have extended our work to show that nucleosomal DNA, extracted from drug-treated nucleosomes, possesses differing electrophoretic mobilities and exhibits different melting behavior to DNA extracted from non-treated nucleosomes. The results are similar to those recently reported studying the effect of *cis*-DDP on isolated supercoiled SV40 DNA [9] as well as earlier experiments employing pSM1 DNA [10] and PM2 supercoiled DNA [11], which report retardation of the *cis*-DDP-treated supercoiled DNA on agarose gels at far shorter incubation times and/or concentrations than those required with the *trans* isomer.

2. MATERIALS AND METHODS

cis-Dichlorodiammineplatinum(II) was purchased from Aldrich. *trans*-Dichlorodiammine-

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platinum(II) was synthesized from potassium tetrachloroplatinate as in [12]. Nucleosomes were prepared from calf thymus using a modification of the procedure in [13]. Five g of calf thymus were homogenized in buffer A [0.25 M sucrose, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1% Triton X-100, 50 mM Tris–HCl (pH 7.4)] and then washed 3 times in buffer A without Triton X-100. The nuclei were suspended in buffer B [10 mM Tris–HCl (pH 7.5), 10 mM NaCl, 3 mM $MgCl_2$, 1 mM PMSF] containing 1 mM $CaCl_2$, washed twice and digested for 30 min with micrococcal nuclease (P-L Biochemicals, 25 units/ml), at a DNA concentration of approx. 10 mg/ml. The reaction was terminated by chilling the tubes and centrifuging for 5 min at 4000 × *g* in a Sorvall HB4 rotor. The pellet was resuspended with a Dounce homogenizer in buffer C [1 mM EDTA, 10 mM Tris–HCl (pH 7.5), 1 mM PMSF], swelled on ice for 20 min and then centrifuged for 20 min at 16000 × *g*. This last step was repeated with the resulting pellet and the two supernatants were combined. The nucleosomes were recovered by adding 0.1 M NaCl and 3 vols ethanol at −20°C. This is the procedure described by authors in [13], employed to fractionate active nucleosomes from trout testes and then employed with calf thymus nuclei to produce a fraction of mono-, di- and trinucleosomes containing H1. We have also used this technique with rat liver and calf thymus to produce H1 containing mono- and dinucleosome fractions which were characterized by sucrose gradient centrifugation, SDS-polyacrylamide gel and agarose gel electrophoresis to produce particles primarily of approx. 200 bp average repeat distance and containing H1 [14]. The nucleosomes were then resuspended into 10 mM sodium phosphate (pH 6), containing 1 mM Na_2EDTA, 1 mM PMSF and 5 mM NaCl. The protein concentration was determined as in [15] employing a calf thymus histone solution as a standard. Nucleosomes were incubated with either *cis*- or *trans*-DDP for differing lengths of time (0.5–2 h). Control mononucleosomes were incubated in parallel, substituting buffer for drug. The drug concentrations varied from 0.03 to 0.3 mM DDP with 0.25 mg/ml of nucleosomal protein. The reactions were quenched and the nucleosomes recovered by adding concentrated NaCl to raise the chloride concentration to 0.2 M and 3 vols ethanol at −20°C.

Proteins were determined as in [15] employing a calf thymus histone solution as a standard. DNA was extracted from a portion of the drug-treated nucleosomes by addition of 400 μl trypsin (1 mg/ml) to 2 ml nucleosome suspension (0.6 mg DNA/ml), and the mixture incubated at 37°C for 60 min. A 10-fold excess of fresh chloroform–isoamyl alcohol (24:1) was added, vortex mixed and allowed to stand at room temperature before being centrifuged at 1000 × *g* for 10 min. The aqueous layer was removed and the DNA precipitated by the addition of 0.2 M NaCl and 3 vols of ethanol at −20°C.

Agarose gel electrophoresis was performed as in [16] and stained with 1 μg/ml of ethidium bromide for 30 min followed by destaining in water overnight at 4°C. The histone proteins were monitored by SDS-polyacrylamide gel electrophoresis using a modification of the procedure in [17]. The stacking gel contained 4.2% (w/v) acrylamide–bisacrylamide (37.5:1, w/w) in 0.1% SDS, 0.125 M Tris–HCl (pH 6.8). The separating gel contained 12% (w/v) acrylamide–bisacrylamide (37.5:1, w/w) in 0.1% SDS, 0.375 M Tris–HCl (pH 8.8) and the reservoir buffer contained 0.1% SDS, 0.19 M glycine, 0.015 M Tris–HCl (pH 8.2). The current was maintained at 7.5 mA until the samples had migrated through the separating gel when it was increased to 12.5 mA until the marker dye was within 0.5 cm of the bottom. The gels were stained with 0.1% (w/v) Coomassie blue in methanol–acetic acid–water (10:7:83, v/v) and destained in the same solvent mixture. Melting curves were performed in a Gilford 250 spectrophotometer with quartz cuvettes. The temperature was increased at 1°C/min from 25 to 90°C by a circulating water bath. Terbium binding curves were performed with the extracted DNA at a constant concentration of nucleic acid-P (50 μM) with increasing terbium concentrations (0–200 μM). The buffer employed was 20 mM Tris–HCl (pH 7.3). An excitation wavelength of 290 nm was used with an emission wavelength of 544 nm in a Perkin Elmer 43A fluorescence spectrophotometer. Platinum determinations were performed using an ILI51 flame photometer equipped with a platinum lamp. The samples were digested in 0.1 N HCl prior to their nucleotide concentration being determined at 260 nm and the platinum concentration

Table 1

Binding of *cis*- and *trans*-DDP to calf thymus nucleosomes

Concentration of DDP added (μM)	Time of incubation (min)	Concentration of bound *cis*-DDP/ μM DNA (*r*)	Concentration of bound *trans*-DDP/ μM DNA (*r*)
0.3	120	0.14 ± 0.03	0.14 ± 0.03
0.075	30	0.07 ± 0.02	0.09 ± 0.02
0.03	30	0.03 ± 0.01	0.04 ± 0.02

Experimental conditions and nucleotide and platinum determinations were determined as described in the text. The nucleosome concentration was constant at 0.25 mg/ml of nucleosomal protein. The error is expressed as ± SE

compared to a standard curve employing a solution of K_2PtCl_4 in 0.1 N HCl.

3. RESULTS AND DISCUSSION

In table 1 is presented the amount of platinum bound/μM DNA-P at different drug concentrations and incubation times with calf thymus nucleosomes. It can be seen that at the lowest concentration and shortest incubation time an *r* value of approx. 0.05 is observed, which increases to approx. 0.15 at the highest drug concentration and longest incubation time employed. These *r* values are higher than those with kidney nucleosomal core particles labelled with radioactive DDP reported in [7].

When the DNA is extracted from the treated nucleosomes and subsequently analyzed on agarose gels, a retardation in mobility as well as a diminution in fluorescence intensity is observed in the fluorescence of the ethidium bromide stain with the *cis*-DDP-treated nucleosomal DNA (see fig.1). The effect is evident only at the high drug concentration and long incubation time (corresponds to $r = 0.15$). The retardation produced by the *cis*-DDP is greater than any effect observed

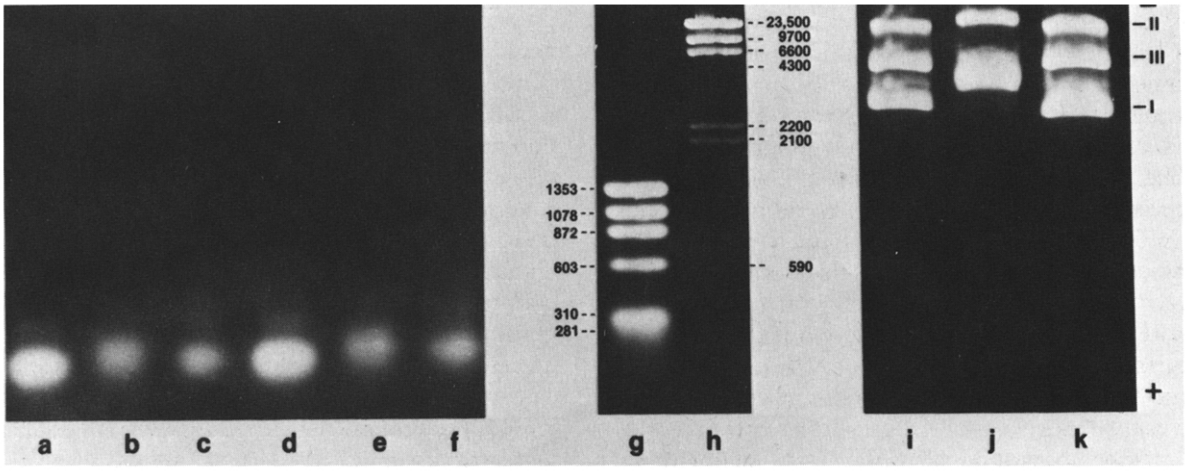

Fig.1. Agarose gels (1.5%) of control nucleosomal DNA (a), DNA from *cis*-DDP-treated nucleosomes (b), DNA from nucleosomes treated with *trans*-DDP (c) and the experiment repeated (d) control, (e) *cis*-DDP- and (f) *trans*-DDP-treated nucleosomes. Experimental conditions are described in the text and the nucleosomal DNA concentration was equal in each lane (4 μg, lanes a–f). M_r markers of *Hin*dIII-digested λDNA (g) and ϕX174 DNA (h) are presented. In addition, from another experiment, pBR322 DNA treated with a similar concentration of *cis*-DDP (i) and *trans*-DDP (j) is presented. (k) Control, non-treated pBR322 DNA.

with *trans*-DDP and is similar to the effect observed with pBR322 DNA incubated with *cis*-DDP (although quantitatively smaller). Authors in [9] reported a diminution in the mobility of SV40 DNA which they ascribed to an unwinding of the DNA. They showed that the effect produced by *cis*-DDP becomes apparent at a binding ratio of approx. 0.08 and with *trans*-DDP at a binding ratio of 0.15. Thus, the effect is observed at far lower amounts of bound drug with supercoiled DNA. It must, however, be pointed out that with supercoiled DNA, a decrease in mobility of form I is directly correlated to its unwinding. Changes in the electrophoretic mobility of linear DNA cannot be explained as easily since unwinding may produce an increase in mobility whereas the formation of higher M_r species, by crosslinking, etc., could result in decreased mobility. However, we have shown [18] that polydisperse linear DNAs are retarded following treatment with *cis*-DDP and that the ethidium bromide fluorescence is diminished whereas that of acridine orange exhibits a red shift suggesting unwinding of the helix. This effect with linear DNA, however, was not as dramatic as that observed with supercoiled DNA.

In addition, we have shown that linear DNA treated with *cis*- and *trans*-DDP [18] results in the helix being disrupted by the former but not the latter, and 'opened-up' regions and/or nicks are produced so that the DNA becomes more accessible to terbium (a fluorescent probe specific for unpaired guanine residues [19,20]). Thus, it was of interest to determine whether this effect was observed with DNA isolated from calf thymus nucleosomes which had been treated with these drugs. The extracted DNA was reacted with terbium – no significant enhancement in fluorescence intensity was observed. The 10-fold enhancement observed following *cis*-DDP treatment of linear DNA (defined *Hin*dIII restriction enzyme fragments and polydisperse calf thymus or *E. coli* DNA) was not observed with DNA isolated from treated nucleosomes [18].

Finally, the nucleosomal DNA was analyzed by its melting behavior at 260 nm (which primarily reflects A-T rich regions), and at 280 nm (which reflects G-C rich regions) (see fig.2). It was found that a marked decrease in melting temperature as well as a broadening of the melting curves was observed with treated nucleosomal DNA and that

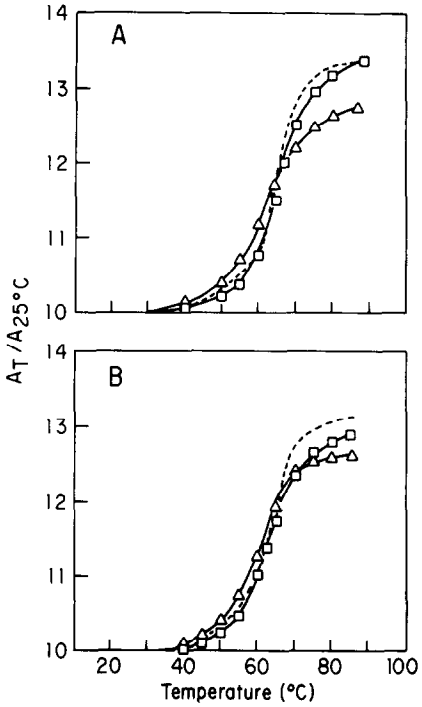

Fig.2. Melting curves performed at 280 nm (A) of DNA extracted from control calf thymus nucleosomes (– – –), *cis*-DDP-treated nucleosomes (Δ—Δ) and *trans*-DDP treated nucleosomes (□—□). Melting curves performed at 260 nm (B) of DNA extracted from control calf thymus nucleosomes (– – –), *cis*-DDP-treated nucleosomes (Δ—Δ) and *trans*-DDP-treated nucleosomes (□—□). The *r* value was 0.15. The solvent was 10 mM Na phosphate (pH 6.0) containing 1 mM PMSF and 1 mM Na_2EDTA.

this effect was apparent even at lower *r* values (0.09–0.11) than those required to produce a change in electrophoretic mobility. In addition, the effect on melting behavior was more apparent at 280 nm than at 260 nm, a result which correlates with the G-C specificity of *cis*-DDP with isolated linear DNA. No effect was observed at the lowest *r* value (0.05) in melting behavior or electrophoretic mobility, although at this level of drug binding changes were observed in the fluorescence intensity of a probe attached to histone H3 [8]. It should be pointed out that melting curves performed with drug-treated whole nucleosomes were frequently found to result in precipitation and thus were suspect. Also, the changes observed with extracted DNA in 10 mM Na^+ were also observed at lower ionic strengths (0.5 mM Na^+).

Our results involve employment of DNA extracted from the drug-treated nucleosome for two reasons. Firstly, terbium titration curves as well as melting curves are suspect when performed with drug-treated nucleosomes due to precipitation problems; secondly, any effects observed with the whole nucleosome can be due to changes within the DNA itself or an effect resulting from an altered DNA–protein interaction. Since it has been shown [7,8] that both drugs react with the nuclear proteins within the nucleosome, it appeared important to be able to separate this effect from that involving solely the nucleosomal DNA. It is interesting to note that our data with DNA extracted from calf thymus nucleosomes parallel those observed with high M_r chromatin threads [21].

It can be argued that these results with drug-treated nucleosomes cannot be related to supercoiled plasmid DNA since the latter is under torsional stress and that relaxation of that stress is normally due to a change in the linking number (resulting in decreased electrophoretic mobility). However, recent evidence suggests that a small proportion of nucleosomal DNA may also be under torsional stress; (a) a small proportion of SV40 minichromatin can be relaxed with topoisomerase I [22]; (b) authors in [23] recently showed that the nuclease hypersensitive domains of globin gene DNA exist in supercoiled but not relaxed plasmid clones in positions corresponding to those activated in chromatin. It thus seems possible (as pointed out in a recent review [24]) that if a structural change takes place in the nucleosome such as the rearrangement of a histone protein then the nucleosomal DNA at that site may be left under torsional strain. Since we [25] and others [26] have shown that *cis*-DDP interacts with histone and non-histone proteins, it is possible that the DNA at the site of interaction may be forced into the supercoiled state. However, these changes may involve only a small percentage of the total DNA, depending on the number of drug binding sites and the length of the DNA region disrupted at each site.

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