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INTERACTION OF *cis*-DIAMMINEDICHLOROPLATINUM (II) WITH SINGLE-STRANDED DNA IN THE PRESENCE OR ABSENCE OF *ESCHERICHIA COLI* SINGLE-STRANDED BINDING PROTEIN

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Abstract—We have compared the mode of fixation *in vitro* of the antitumor drug *cis*-diamminedichloroplatinum (II) (*cis*-DDP) to single-stranded M13mp10 DNA either in the presence or absence of the *Escherichia coli* single-stranded binding protein (SSB). Platinum binding sites have been identified by taking advantage of their capacity to inhibit DNA replication of primed M13 DNA catalysed by *E. coli* DNA polymerase I large fragment. We report here that the presence of SSB increases the number of platinum–DNA lesions and alters their distribution. We also present evidence that SSB allows *cis*-DDP to bind to DNA sequences otherwise less accessible.

The antitumor compound *cis*-diamminedichloroplatinum (II) (*cis*-DDP) interacts with DNA both *in vivo* and *in vitro* [1] and this interaction is believed to be responsible for the cytotoxic effect of the drug [2]. A great deal is known about the lesions formed by *cis*-DDP on DNA. The drug binds primarily to adjacent bases in GpG and ApG sequences [3]. These data have been obtained by two approaches: (a) direct quantification of the adducts by physical and chemical methods [4, 5]; (b) determination, via DNA sequencing techniques, of either arrest sites of DNA synthesis on single-stranded DNA [6, 7] or sites of inhibition of exonuclease III action on double-stranded DNA [8]. In all these studies the drug was allowed to interact with DNA which was free of proteins.

The *Escherichia coli* single-stranded binding protein (SSB) has a great affinity for single-stranded DNA and plays an essential role in DNA replication, recombination and repair [9–11]. This protein appears to have the capacity of extending single-stranded DNA *in vitro* by removing regions of secondary structure (hairpin) and to organize it in nucleosome-like units [12, 13]. Therefore we decided to investigate its effect on the fixation of *cis*-DDP to single-stranded M13 DNA. We report here that the number and distribution of the drug–DNA lesions (which we have identified via their capacity to inhibit DNA synthesis by *E. coli* polymerase I Klenow fragment) is different when the DNA is exposed to *cis*-DDP in the presence or absence of SSB.

MATERIALS AND METHODS

Reagents and enzymes. cis-DDP was synthesized following published methods [14] and provided by Dr N. P. Johnson. DNA polymerase I (Klenow fragment) was from Pharmacia (Uppsala, Sweden); one unit of activity is the amount that catalyses the incorporation of 10 nmol dNTPs into acid insoluble material in 30 min at 37°. Synthetic oligonucleotides primer (17 mer), unlabelled dNTPs, dideoxy NTPs and single-stranded binding protein (SSB) of *E. coli* were from Pharmacia LKB Biotechnologies Inc; α-^{32}P-labelled dATP was from Amersham (Amersham, U.K.). Collodium membranes were purchases from Schulher and Schull (Keene, NH, U.S.A.). Proteinase K was from the Sigma Chemical Co. (St. Louis, MO, U.S.A.).

DNA preparation and treatment with cisplatinum in the presence or absence of SSB. M13mp10 DNA was prepared as described previously [15]. Synthetic oligonucleotide was annealed to the template by heating the phage DNA with primer (2 ng primer/μg DNA) at 60° for 1 hr.

Typically, 7 μg of primed single-stranded DNA in 20 μL of Tris–HCl buffer were incubated 15 min at 37° with 16 μL 20 mM Tris–HCl (pH 7.9), 0.1 mM EDTA, 50% glycerol, 0.1 mM dithiothreitol and 0.2 M NaCl, containing or not 50 μg *E. coli* SSB. This concentration of SSB was chosen because the ratio (w/w) of approximately 7:1 of SSB over DNA is known to allow the complete covering of the template by the protein [16]. The samples were then treated with a 0.9% KCl solution of freshly made *cis*-DDP at a drug:nucleotide ratio (R_i) of 2, for 40 min at 37°. To stop the platinum reaction, NaCl was added to a final concentration of 0.5 M and samples immediately placed on ice. To remove unreacted drug, samples were subsequently dialysed for at least 18 hr at 4° against 10 mM Tris–HCl (pH 7.5) and 1 mM EDTA containing 0.5 M NaCl.

In order to eliminate SSB protein, samples were

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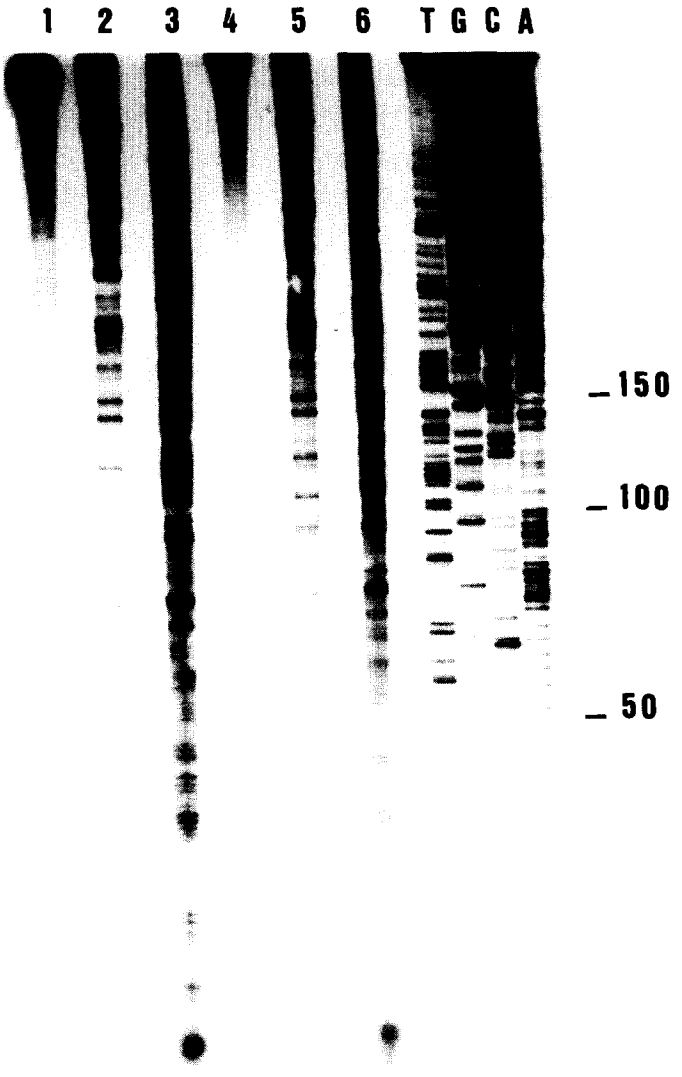

Fig. 1. Comparison of the products of DNA synthesis by *E. coli* DNA polymerase I on primed single-stranded DNA treated in the presence or absence of SSB. M13 single-stranded DNA was incubated 15′ at 37° with or without *E. coli* SSB as described in Materials and Methods. The polymerase I assay was performed using 1 unit of enzyme and the products of DNA synthesis were analysed by polyacrylamide gel electrophoresis. Lane 1, untreated DNA; lane 2, DNA treated in absence of SSB; lane 3, DNA treated in presence of SSB; lanes 4, 5, 6, same substrates as lane 1, 2, 3 after proteinase K digestion and phenol extraction. A, C, G, and T show the result of Sanger sequencing reactions. The sequencing lanes are labelled according to the template strand for direct comparison to the platinated template. Nucleotide numbering is from the 3′ OH end of the synthetic primer.

treated with Proteinase K (55 μg/mL) for 2 hr at 37° and primed DNA was extracted twice with phenol.

Quantification of platinum bound to DNA. The DNA concentration was determined by UV absorbance and the platinum bound to the DNA measured using a Perkin–Elmer atomic absorption spectrophotometer model 603 equipped with a graphite furnace (limit of detection 100 pg/20 μL). The molar ratio of platinum covalently bound: nucleotide (R_b) was then calculated.

DNA polymerase reaction. The reaction mixture (10 μL) contained 50 mM Tris–HCl (pH 7.5), 6 mM $MgCl_2$, 0.5 mM dithiothreitol, 50 mM NaCl, 25 μM of dCTP, dGTP and dTTP, 2.5 μM [^{32}P]dATP (10,000 cpm/pmol), 200 ng of DNA untreated or *cis*-DDP treated in the absence or presence of SSB and 1 unit of polymerase I; the reaction mixture was incubated at 37° for 30 min; at the end of the reaction samples were placed on ice, aliquots spotted on Whatman glass filters (GF/C) and the remaining samples immediately frozen in liquid nitrogen and stored at −20° for subsequent DNA sequencing. Filters were washed with ice-cold 5% trichloroacetic acid, 1% sodium pyrophosphate, then with ethanol and the radioactivity determined.

Electrophoresis analysis of DNA synthesis

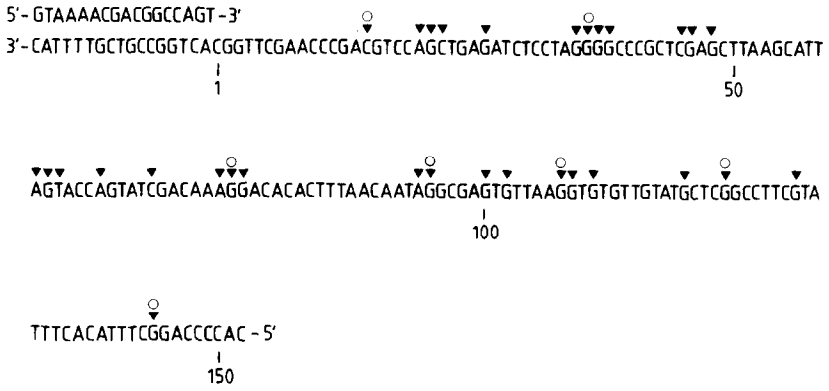

Fig. 2. Schematic diagram showing the portion of sequence (platinated strand) used to monitor inhibition of DNA synthesis by *cis*-DDP. Nucleotide numbering is from the 3′ OH end of the synthetic primer. Arrest sites have been drawn based on several autoradiographies of the gel shown in Fig. 1, which was exposed for different times. (○) Termination bands detected on DNA platinated in the absence of SSB; (▼) termination bands detected on DNA platinated in the presence of SSB.

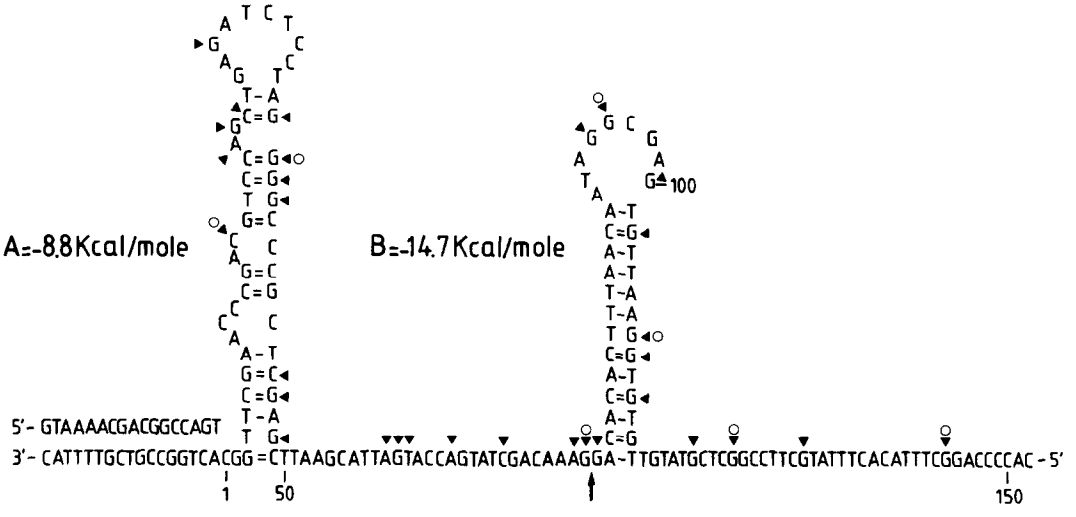

Fig. 3. Potential short-range secondary structure interactions. Along the 150 nucleotides of M13mp10 downstream the 3′ OH end of the primer, the two stems which present a significant stability (free-energy $G^0 < -5$ Kcal/mol) are identified by a capital letter (A and B) and their respective folding energy given. M13mp10 sequence is numbered from the 3′ OH end of the primer. The arrow indicates the strong pausing site detected during the replication of this sequence by the eukaryotic α DNA polymerases. Symbols are as in Fig. 2.

products. Electrophoresis was carried out not longer than 24 hr after DNA synthesis. One volume of deionized formamide containing 0.3% xylene cyanol, 0.3% bromophenol blue, and 0.3% EDTA was added to DNA samples which had the same amount of radioactivity; samples were boiled for 5 min and then subjected to electrophoresis at 1500 V on an 8% polyacrylamide, 7 M urea denaturating gel. Autoradiography of the gels was performed using Trimax 3M film at −70°. Dideoxy sequencing reactions [17] were carried out on untreated M13mp10 DNA.

Computer prediction of secondary structure. We searched for locally stable secondary structure interactions in the 150 nucleotides of the M13mp10 single-stranded DNA, immediately downstream of the 3′ OH of the primer. This analysis was performed using the Zuker's RNA fold program [18] in conjunction with the thermodynamic parameters determined by Freier *et al.* [19]. In the first step the entire region had been globally folded, then a scan was performed with an 80 nucleotides window and a 20 nucleotides step to emphasize short-range interactions.

RESULTS

E. coli *single-stranded binding protein modulates the fixation of* cis-*DDP to single-stranded DNA.*

We have investigated the fixation of *cis*-DDP to

single-stranded DNA under conditions in which it was either uncovered or covered with single-stranded binding protein. In a typical experiment, 7 μg of primed M13 single-stranded DNA were incubated 15 min at 37° with 50 μg pf *E. coli* SSB in a solution containing a final NaCl concentration of 80 mM; the DNA was treated with *cis*-DDP for 40 min at 37° at an $R_i = 2$ and then unreacted drug was eliminated by extensive dialysis against buffer containing 0.5 m NaCl. In parallel, 7 μg of the same DNA were treated with *cis*-DDP under identical conditions, but in the absence of *E. coli* SSB, and the two substrates were replicated with 1 unit of *E. coli* DNA polymerase I. As measured by acid precipitable material, the residual synthesis was 45% in the case of DNA treated in the absence of SSB and 5% for DNA treated in the presence of SSB. SSB alone did not exercise any inhibitory effect on DNA polymerase I on intact template nor did it alter the inhibitory effect of *cis*-DDP lesions when added after the treatment of DNA (data not shown). The products of DNA synthesis were anlysed by polyacryclamide gel electrophoresis (Fig. 1). Lane 1 shows the pattern of DNA synthesis on untreated DNA. A similar pattern was observed on untreated DNA covered by SSB (data not shown). On the other hand, the pattern of arrest sites detected upon replication of DNA treated with *cis*-DDP was different in the absence and in the presence of SSB. As can be seen in Fig. 1, more arrest sites are detected in the latter case (compare lanes 2 and 3), thus confirming the increased inhibition observed by analysis of the acid precipitable material.

The stronger inhibition observed in the presence of SSB could have been due either to an increased number of *cis*-DDP–DNA adducts or to a direct interaction of *cis*-DDP with *E. coli* SSB which cover the substrate. In order to test this second possibility, we subjected the two DNAs to treatment with proteinase K followed by phenol extraction (see Materials and Methods) and a new assay of DNA synthesis was performed. Again, we found a much larger inhibition of DNA synthesis on the phenol extracted DNA initially treated with *cis*-DDP in the presence of SSB compared to the DNA phenol extracted after treatment with the drug in the absence of SSB. Furthermore, phenol extraction of SSB did not change the pattern of arrest sites (compare lanes 4 to 1, 5 to 2 and 6 to 3 in Fig. 1), strongly suggesting that *cis*-DDP–DNA adducts are responsible for the novel arrests sites observed following treatment of the DNA when covered by SSB. This was confirmed by a direct quantification of the amount of *cis*-DDP bound to the DNA (see Materials and Methods). The average number of *cis*-DDP–DNA adducts, determined in three independent experiments from phenol extracted substrates, was of 1 adduct per 500 nucleotides in the case of DNA treated in the absence of SSB and 1 adduct per 250 nucleotides of DNA treated in the presence of SSB.

Sequence localization of the arrest sites

We have localized most of the termination sites detected on *cis*-DDP treated primed M13 DNA over a region of approximately 150 nucleotides from the 3′ start of DNA synthesis. As can be seen from Fig. 2, the arrest sites detected on the template platinated in the absence of SSB are located either at a base preceding or at a site of putative *cis*-DDP lesions (i.e. mainly GG, AG or CG sequences) as reported previously [6, 7]. Moreover, the additional arrest sites obtained following treatment of the template in the presence of SSB are also located at sites of putative *cis*-DDP–DNA lesions, suggesting that the action of SSB protein renders these sequences more accessible to the drug.

Correlation between arrest sites and secondary structure

We investigated the existence of regions of secondary structure in the 150 nucleotides long sequence on which the arrest sites have been mapped. As can be seen in Fig. 3, two major hairpin structures are predicted on the basis of thermodynamics rules, one spanning from nucleotide 3 to 50 (stem A) and the other from nucleotide 79 to 113 (stem B). These two hairpins present different stability, with only −8.8 Kcal/mol for stem A, which presents several irregularities and −14.7 Kcal/mol for stem B. Accordingly, we always detected the existence of stem B even if secondary structure prediction was performed over a segment of 280 nucleotides. It is worth noting that more sites of termination induced by SSB are detected in the region of stem A (compare lanes 5 and 6 of Fig. 1).

DISCUSSION

Many data indicate that the antitumor drug cisplatinum exerts its toxic action by inhibiting DNA replication [6, 7, 20]. Single-stranded binding proteins, such as *E. coli* SSB, play a key role in replication, recombination and repair of DNA; they act by extending and protecting the transient single-stranded DNA regions, and in such a way, they might contribute to the processivity and fidelity of DNA polymerases [9–11]. In this study we have investigated the mode of fixation of the antitumoral drug cisplatin to either "naked" single-stranded DNA or to the same DNA covered by SSB protein.

Platinum binding sites have been identified by taking advantage of their capacity to inhibit *in vitro* replication catalysed by the *E. coli* DNA polymerase I Klenow fragment [6, 7]. As measured by acid precipitable material, this inhibition was of 95% in the case of DNA treated with *cis*-DDP in the presence of SSB and of 55% for DNA treated with *cis*-DDP under identical conditions but in the absence of SSB. Polyacrylamide gel analysis of the products showed that the appearance of new arrest sites on the template DNA corresponded to this increased inhibition of replication (compare lanes 2 and 3 of Fig. 1).

In addition to binding to nucleic acids, *cis*-DDP is known to react with proteins and to form protein–DNA crosslinks [21, 22]. We therefore asked the question of whether these new arrest sites were due to additional *cis*-DDP–DNA adducts, produced in the presence of SSB, or to crosslinks of the drug either with the protein alone or between SSB and single-stranded DNA. In an attempt to elucidate this point, we treated the two templates with

proteinase K followed by phenol extraction and subsequently we performed a new assay of DNA synthesis. As can be seen in Fig. 1, the arrest sites detected were almost identical to the one obtained prior to phenol extraction (compare lanes 5 to 2 and 6 to 3). Furthermore, direct quantification of the adducts on phenol extracted DNAs showed twice as many *cis*-DDP adducts in the DNA treated with the drug in the presence of SSB. Taken together, these data strongly suggest that the additional lesions formed by *cis*-DDP on single-stranded DNA in the presence of SSB are platinum–DNA adducts, although the persistence of some residual crosslinks between DNA and SSB, which might be resistant to proteinase treatment and phenol extraction, cannot be completely ruled out. A similar conclusion (i.e. preferential binding of ciplatinum to DNA in the presence of proteins) has been suggested in previous studies investigating the *in vitro* binding of *cis*-DDP with either nucleosome core particles or chromatin [23, 24].

A mapping of most of the arrest sites detected, either in the presence or absence of SSB, over a region of 150 nucleotides starting from the 3′ OH primer of the template is shown in Fig. 2. One can see that almost all the termination bands are localized on sequences already known to be putative sites of interaction between *cis*-DDP and single-stranded DNA [6, 7], suggesting that SSB would act by facilitating the binding of *cis*-DDP to a region of DNA less accessible to the drug in its absence. SSB is known to form a nucleosome-like structure with 145 nucleotides wound around an SSB octamer and a 30 nucleotide linker sequence [12]; it is therefore possible that additional binding sites are available in the protein–DNA complex. On the other hand, since *E. coli* single-stranded binding protein has been reported to be able to extend single-stranded DNA in solutions containing up to 0.2 M NaCl [12], we investigated the existence of regions of secondary structure in the 150 nucleotides long sequence on which the arrest sites have been mapped. Two major hairpin structures are revealed by the method of calculation we have employed, one spanning from nucleotide 3 to 50 and the other from nucleotide 79 to 113 (Fig. 3). To this respect, it is interesting to note that, in the course of previous studies, we have routinely observed (see for instance figure 4 of Ref. 25) a very strong pause site during the replication of M13mp10 DNA template by α DNA polymerases, whose mode of replication is known to be particularly sensitive to the presence of secondary structure in the template [26, 27]; this pausing site is located precisely at a GG sequence immediately preceding the stem B, which is the second and more stable hairpin depicted in Fig. 3 (see arrow). From this picture it appears that another possible explanation accounting for the increased number and different distribution of *cis*-DDP–DNA lesions detected in the presence of SSB would be the capacity of the protein to melt such hairpin structures. This process would allow the drug to reach DNA sequences otherwise less accessible. In this respect, it is interesting to note that almost all of the arrest sites detected in the absence of SSB are localized at or near GG sites (see Figs 1 and 2); it is therefore possible that sites which are not available for platinum binding when part of secondary structures are those other than GG sites. The fact that the secondary structure present from nucleotide 3 to 50 appears to be energetically less stable than the other one might facilitate its disruption by SSB, thus explaining the appearance of more SSB induced lesions in this region of the sequence, as shown in Fig. 1. Whatever the mechanism, our results indicate that the number and distribution of platinum adducts are altered in SSB–DNA complexes; given the role attributed to single-stranded binding proteins in replication, recombination and repair [9–11] this finding might have some implications on the genotoxic effect of the drug.

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