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Effects of a single intrastrand d(GpG) platinum adduct on the strand separating activity of the *Escherichia coli* proteins RecB and RecA

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RecB and RecA proteins play key roles in the process of DNA recombination in *Escherichia coli* and both possess DNA unwinding activities which can displace short regions of duplex DNA in an ATP-dependent manner in vitro. We have examined the effect of the most abundant DNA adduct caused by the chemotherapeutic agent *cis*-diamminedichloroplatinum(II) on those activities. For this purpose, we have constructed a partially duplex synthetic oligonucleotide containing the intrastrand d(GpG) crosslink positioned at a specific site. We report here that both the DNA strand separating and DNA-dependent ATPase activities of the RecB protein are inhibited by the d(GpG) *cis*-DDP adduct. In contrast, neither the unwinding nor the ATPase activities of RecA protein appear to be perturbed by this lesion.

Escherichia coli RecA and RecB proteins; DNA helicase activity; *cis*-Diamminedichloroplatinum(II) d(GpG) adduct

1. INTRODUCTION

The interaction with DNA of the antitumor drug *cis*-diamminedichloroplatinum(II) (*cis*-DDP or cisplatin) is thought to be responsible for its toxicity and mutagenicity [1]. Among the DNA adducts formed by *cis*-DDP, the 1,2 intrastrand d(GpG) crosslink is the most abundant both in vivo and in vitro [2,3]. This lesion represents a strong (although not absolute) impediment for the in vitro elongation catalyzed by DNA and RNA polymerases [4–7], is poorly repaired by purified *E. coli* UvrABC enzyme [8] and appears to be refractory to repair by human cell extracts [9,10].

Previous research on the mechanism of action of cisplatin in vitro has mainly investigated the consequences of the d(GpG) *cis*-DDP crosslink on DNA replication, transcription or repair [4,7,9]. We thought that it would be of interest to examine the effect of this adduct on the helicase activities of two proteins, RecB and RecA, which are necessary for recombination of DNA in *E. coli*. Genetic experiments have shown that wild- type RecA and RecB proteins participate in cellular processes that protect *E. coli* against the toxic and mutagenic effects of cisplatin [11]. RecA is required for homologous recombination and for repair of single- stranded breaks in DNA [12], while RecBCD plays a key role in the intermolecular events of recombination and in the repair of blunt double-stranded breaks [13]. RecA protein is able to bypass UV DNA damage and carry out strand exchange in vitro [14]. The influence of cisplatin lesions on RecA or RecBCD activities is unknown. In order to investigate the effect of cisplatin on homologous DNA repair we have carried out a preliminary study on the unwinding activities of RecA and RecB. Biochemical and genetic evidences indicate that RecBCD enzyme initiates recombination by nicking DNA and subsequently unwinding the duplex to produce single-stranded DNA. RecA protein then promotes homologous pairing of this ssDNA with duplex DNA and DNA strand exchange [15]. The RecBCD protein displays a non-specific nuclease activity. Hence we have examined the effect of cisplatin on the unwinding activity of RecB, the helicase subunit of this enzyme [16].

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2. MATERIALS AND METHODS

2.1. *Buffers*

RecA unwinding buffer was 30 mM Tris-HCl pH 7.5, 12 mM $MgCl_2$, 0.5 mM dithiotheitol, 4% glycerol, 2.5 mM ATP. An ATP regeneration system, including 6 mM creatine phosphate and 160 units/ml creatine phosphokinase, was also present. RecB buffer was 25 mM MOPS-KOH pH 7, 10 mM $MgCl_2$, 1 mM dithiotreitol, 100 mg/ml BSA, 2.5 mM ATP. The ATP regeneration system was also included. Helicase stop buffer was 100 mM EDTA pH 8, 30% glycerol, 1% SDS and 0.1% (w/v) Bromophenolblue.

2.2. *Proteins*

RecA was purified to near homogeneity from the *lexA*(Def)*ΔrecA* strain PC1421 [17] harbouring the plasmid pFL352 [18]. Purification was carried out as previously described [19] except that bacteria were grown to saturation without addition of nalidixic acid. RecA concentration was determined both by Lowry's procedure and by absorbance at 280 nm using an extinction coefficient of $A_{280} = 0.59$ ml · mg^{-1} · cm^{-1} [20]. RecB was purified to near homogeneity as published [21]. The concentration of RecB protein was determined by using an extinction coefficient of 164,150 M^{-1} · cm^{-1}, as calculated from the published amino acid sequence [22]. Phosphocreatine kinase (from rabbit mus-

cle), and *Hae*III restriction nuclease were purchased from Boehringer Mannheim. T4 polynucleotide kinase was from USB.

2.3. *Chemicals*

cis-Diamminedichloroplatinum(II), 99.99% pure was purchased from Aldrich Chemical Company and solutions were prepared in 5 mM sodium perchlorate just before use. Creatine phosphate was from Boehringer Mannheim. [^{32}P]ATP was purchased from Amersham.

2.4. *DNA substrates: cis-DDP treatment, 5′ end labeling and annealing*

The oligonucleotides shown in Table I (53- and 23-mers) were synthetised using an Applied Biosystems Synthetiser and purified by electrophoresis on a denaturing polyacrylamide gel. Aliquots (30-50 μg) of the purified 53-mer were resuspended in 5 mM Tris-HCl pH 7.5, 0.5 mM EDTA and incubated with *cis*-DDP at a molar ratio of 1.5 Pt/53-mer for 48–72 h at 37°C. At the end of the incubation, the 53-mer was ethanol- precipitated twice and resuspended in 10 mM Tris-HCl pH 7.5, 0.5 mM EDTA. 5′-OH extremities of the purified 23-mer oligonucleotide were labeled with T4 polynucleotide kinase in the buffer supplied by the manufacturer. First, the oligomer (typically 2000 pmol of 23-mer) was incubated with 200 units of kinase, 50 pmol of [^{32}P]ATP and 250 pmol of cold ATP at 37°C for 20 min. Subsequently, in order to phosphorylate the majority of the available 5′-OH end, the reaction was chased by addition of 2000 pmol of cold ATP, supplemented with 50 more units of kinase and the incubation continued for an additional 20 min. The reaction mixture was then heated at 70°C for 10 min to inactivate the kinase. Under these conditions, we routinely incorporated more than 60% of the labeled [^{32}P]ATP, as judged by acid precipitation of the product. Annealing of the 53- and 23-mers was achieved in the following way: a 1.3 molar excess of either untreated or *cis*-DDP treated 53-mer was mixed with the ^{32}P labeled 23-mer in 50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 150 mM NaCl; the solution was heated at 85°C for 10 min and slowly cooled to 4°C. The annealed material was subsequently ethanol-precipitated and resuspended in 10 mM Tris-HCl pH 7.5, 0.5 mM EDTA.

2.5. *Purification of the* cis-*DDP treated DNA substrate.*

In a standard preparation of substrate (see Table I), 20 μg of 53/23 duplex DNA were incubated with 800 units of *Hae*III restriction enzyme in 10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM dithiotreitol and 50 mM NaCl for 45 min at 37°C in a final volume of 180 μl. Then, 400 more units of restriction enzyme were added and the incubation continued for another 45 min. The reaction was stopped by adding 1/4 of the volume of helicase stop buffer and electrophoresed through a 20% native polyacrylamide gel at 15 V/cm for 2 h. The band corresponding to the restriction enzyme resistant material (see Fig. 1) was identified by autoradiography, cut out from the gel and eluted at 37°C with agitation in 2 ml of 10 mM Tris-HCl pH 7.5, 0.3 M NaCl for at least 24 h. The eluted material was ethanol precipitated in presence of 20 μg of glycogen, centrifuged and resuspended in 10 mM Tris-HCl pH 7.5, 0.5 mM EDTA. Routinely, 30–50% of the DNA substrate loaded on the gel was recovered. Control experiments showed no difference in the capacity of unplatinated substrate to act as a substrate for RecB and RecA strand-separating activities when subjected to this treatment. At the end of each preparation, the concentration of the partially duplex DNA substrates was determined in duplicate by UV absorption (A_{260} = 1 corresponding to 33 μg/ml).

2.6. *RecB and RecA catalysed unwinding of DNA duplex*

Assays were performed in either RecB or RecA unwinding buffers at 30°C for the times, substrates, protein concentrations and volumes indicated in the legends of the figures. Reaction buffers always included a 10 times molar excess of unlabeled 23-mer (the same as the labeled one) as a trap to prevent the released, labeled oligonucleotide from reannealing. Reactions were stopped by the addition of 1/4 volume of helicase stop buffer and the products electrophoresed for 2 h at 15 V/cm on a 20% native polyacrylamide gel prepared in TBE. Gels were dried on DE-81 paper, the radioactive DNA visualized by autoradiography, and the autoradiogram used to identify the position of both the duplex DNA and the displaced fragment on the gel. The corresponding sections of the gel were removed and counted in a Beckman scintillation counter. Unwinding activity was expressed as a percentage of displaced fragments and values were corrected for background by substracting the released fragment obtained in control incubations without either protein or ATP. Typically, such background represented no more than 15% of the total radioactivity for incubation times of 30 min.

2.7. *RecB and RecA ATPase activities*

The DNA-dependent ATPase activities of RecB and RecA proteins were measured according to the spectrophotometric method described previously [23]. The hydrolysis of ATP is linked to the oxydation of NADH by the combined action of pyruvate kinase and L-lactic dehydrogenase in the presence of phosphoenolpyruvate (PEP). Briefly PEP is converted to pyruvate by pyruvate kinase when ADP is regenerated to ATP. Then pyruvate is transformed to lactate by L-lactic dehydrogenase upon oxydation of NADH. The ATPase rate is thus measured by following the reduction of absorbance at 340 nm related to the oxydation of NADH [24]. The turnover of ATP (k_{cat}) is equal to the ATPase rate divided by the protein concentration. Reaction conditions for the DNA-dependent ATPase were identical to those for DNA unwinding. PEP was 3 mM and pyruvate kinase and lactic dehydrogenase were 20 U/ml.

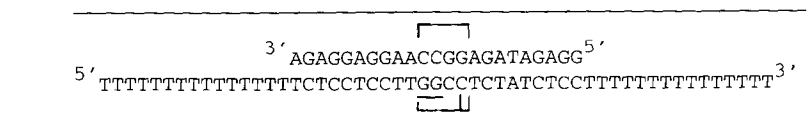
3. RESULTS

3.1. *Purification of the DNA substrate containing the d(GpG) adduct*

A 53-mer oligonucleotide and a complementary 23-mer were synthesized for the purpose of this investigation (see Table I). The sequence of the 53-mer contained a single d(GpG) residue, which is part of the *Hae*III restriction site; no other putative target sequences for *cis*-DDP binding, such as d(ApG) or d(GpXpG) [3], were present. Recently the existence of a *cis*-DDP d(GpC) adduct has been reported, but it appeared to be a very minor one and the authors attributed its formation to an interstrand crosslink [25].

Table I

DNA substrate used in this study



Underlined GG indicate the target sequence for *cis*-DDP binding. The box shows the *Hae*III site present in the template

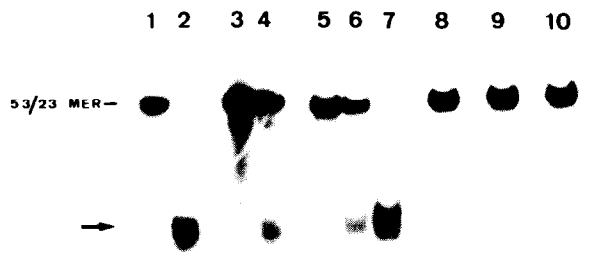


Fig. 1. Structure and purification of the *cis*-DDP treated DNA substrate. The purified 53-mer was *cis*-DDP treated for 72 h at 37°C, annealed to the complementary 23-mer and digested with *Hae*III restriction endonuclease as described in section 2. Lane 1, untreated DNA substrate; lane 2, untreated DNA substrate digested with *Hae*III; lanes 3 and 4, as lanes 1 and 2 but with *cis*-DDP treated DNA; lanes 5 to 7, 100 ng of untreated gel-purified DNA substrate incubated for 1 h at 37°C with 0, 1 and 5 units of *Hae*III, respectively; lanes 8 to 10, 100 ng of *cis*-DDP treated gel-purified DNA substrate incubated for 1 h at 37°C with 0, 1 and 5 units of *Hae*III, respectively. The arrow indicates the position of the *Hae*III digestion product.

Fig. 1 shows the different steps in the purification of the platinated substrate: the 53 oligomer was treated with *cis*-DDP at a ratio of 1.5 mol of platinum per mol of 53-mer for 72 h at 37°C, followed by ethanol precipitation and annealing to the radioactive, complementary 23-mer. The substrate was then subjected to *Hae*III digestion. It is known that *cis*-DDP adducts, when placed in a restriction site, render the DNA sequence resistant to cleavage by the corresponding nuclease [4,9]; consequently the majority of the cisplatin treated substrate was found to be resistant to *Hae*III cleavage, under conditions in which almost 100% of the untreated substrate was digested (see Fig. 1, lanes 1–4). We favour the hypothesis that the small quantity of digested material (see lane 4, Fig. 1) is derived from the cleavage of molecules containing no lesion; supporting this view is the observation that the amount of the digested material appeared to increase when the 53 oligomer was treated with cisplatin for 48 h instead of 72 (data not shown). Kinetic studies of *cis*-DDP adducts formation performed on single-stranded DNA exposed to *cis*-DDP for periods up to 26 h, showed that only d(GpG) and d(ApG) lesions were formed (see Fig. 3 of ref. [5]).

The *Hae*III-resistant material was then purified as described in section 2 and shown to be completely insensitive to the enzyme cleavage (Fig. 1, lanes 9 and 10). Taken together, these experiments strongly suggest that nearly 100% of the cisplatin treated, purified substrate molecules, contained a d(GpG) adduct, which is likely to be the unique one present.

3.2. *Effect of the d(GpG) adduct on the unwinding activity of the* E. coli *RecB protein*

The RecBCD protein of *E. coli* is a multifunctional, multisubunit enzyme which performs critical functions in homologous genetic recombination and is endowed with a wide range of activities, such as ATP dependent exo- and endonuclease, ATP-dependent helicase and DNA-dependent ATPase ([26,27] and references therein).

Of the three subunits, RecB is the sole possessing both ATPase and helicase activites and might be considered the 'engine' of the RecBCD holoenzyme [16]. In vitro, nearly all helicases prefer to initiate unwinding of duplex DNA possessing a flanking single-stranded DNA region [28], therefore we thought that the partially duplex oligonucleotide depicted in Table I would be a suitable substrate for the protein. At first, we performed kinetic experiments to investigate the effect of a d(GpG) *cis*-DDP adduct on strand displacement catalyzed by RecB (Fig. 2). In order to obtain a detectable displacement of the labeled 23-mer, an excess of cold 23-mer had to be present in the reaction to prevent immediate reannealing of the labeled oligonucleotide. We found that a 10 times molar excess of cold 23-mer was necessary to achieve a complete displacement (data not

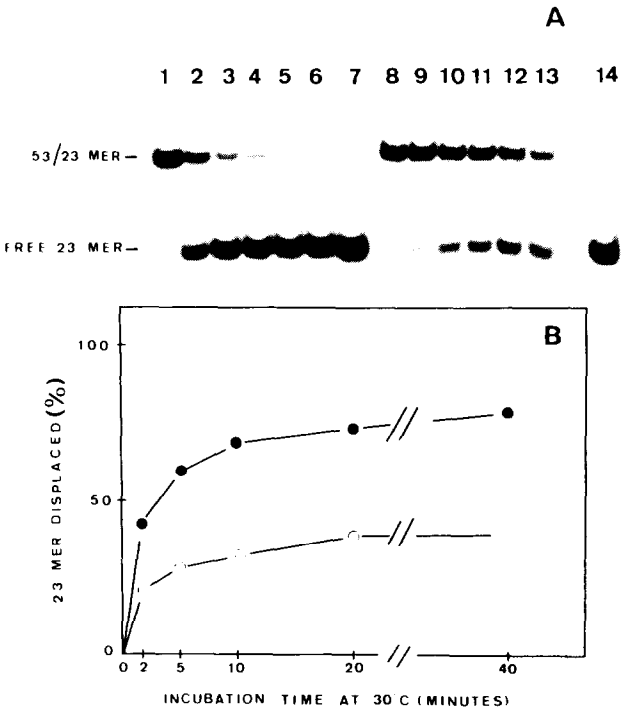


Fig. 2. Effect of the d(GpG) *cis*-DDP adduct on the helicase activity of the RecB protein: kinetics of DNA unwinding. Intact or *cis*-DDP treated DNA substrate (77 nM, oligonucleotide) was incubated at 30°C with 25 nM RecB protein in a total volume of 40 μl containing RecB protein buffer and a tenfold excess of unlabeled 23-mer. At times indicated, 5 μl aliquots were taken and the percentage of 23-mer released determined by polyacrylamide gel electrophoresis as described in section 2. (A) Autoradiogram showing the reaction products: lanes 1 to 7, untreated DNA substrate; lanes 8 to 14, *cis*-DDP treated DNA substrate. Lanes 1 to 6 and 8 to 13: 0, 2, 5, 10, 20 and 40 minutes time points, respectively. Lanes 7 and 14: heat denatured substrates. (B) Quantification of the data shown in (A): sections corresponding to the radioactive bands shown in Fig. 2A were excised from the gel and processed as described in section 2. The amount of displaced 23-mer was calculated as a percentage of the heat denatured control. Calculations made as percentage of the total radioactivity for each time point gave comparable values. Untreated DNA, filled circles; *cis*-DDP treated DNA, open circles.

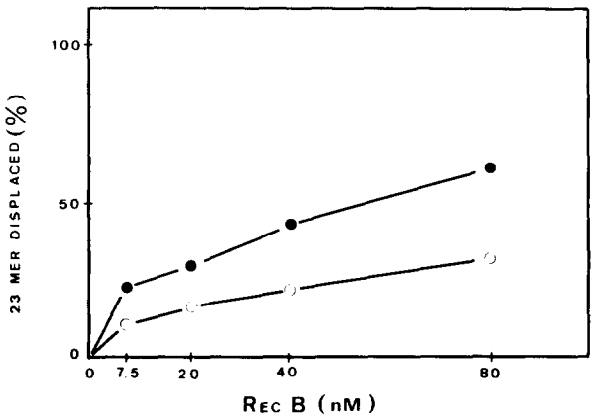


Fig. 3. Effect of the d(GpG) *cis*-DDP adduct on the helicase activity of the RecB protein: protein titration. Intact or *cis*-DDP treated DNA substrate (160 nM, oligonucleotide) was incubated at 30°C with given concentrations of RecB protein in RecB protein buffer and a tenfold excess of unlabeled 23-mer. After a 5 min incubation, the reactions were stopped and the percentage of displaced 23-mer determined as described in the legend to Fig. 2. Untreated DNA, filled circles; *cis*-DDP treated DNA, open circles.

shown); the same requirement was reported for experiments investigating the unwinding of partially duplex oligonucleotides by other prokaryotic helicases [29,30]. As can be seen from Fig. 2A and B, the d(GpG) *cis*-DDP lesion decreased the unwinding activity of RecB. The pattern of inhibition shown in Fig. 2 has been reproduced with four different preparations of the partially duplex substrate. The mean of three independent time course experiments, performed with one substrate preparation, led us to estimate that RecB activity is reduced by 40% under these experimental conditions. Addition of fresh enzyme after 15 min reaction did not change the reaction rate on the platinated substrate. On the other hand, addition of fresh substrate during the course of the reaction led to increased strand displacement, indicating that the enzyme remains active (data not shown).

Finally, we investigated the effect of varying the concentration of RecB protein on its capacity to unwind *cis*-DDP treated or untreated substrate. The result shown in Fig. 3 indicates that the extent of inhibition of the RecB activity by the d(GpG) adduct remained roughly constant throughout a ten fold range variation of the protein concentration. Even at an excess molar ratio of protein over substrate comparable to conditions used for RecA (160 nM RecB for 20 nM substrate), we observed about 40% inhibition of RecB unwinding activity by the lesion (data not shown).

3.3. *Effect of the d(GpG) adduct on the unwinding activity of the* E. coli *RecA protein*

RecA protein plays a key role in the recombination process of *E. coli* and catalyzes DNA strand exchange in vitro [31]. RecA has been shown to possess a DNA unwinding activity, although limited to the displacement of short duplexes [32]. Accordingly, we found here that RecA could displace, in an ATP-dependent manner, the 23 oligonucleotides from the duplex DNA substrate (Fig. 4) although it did so less efficiently than RecB under our experimental conditions. In fact, roughly 700 nM RecA protein versus 25 nM RecB protein was required to displace the same amount of substrate (compare Figs. 2 and 4), suggesting that RecA protein is required in stoichiometric amounts. As for RecB, reactions were run in presence of 10 molar excess of cold 23-mer. Kinetic experiments aimed at investigating the effect of the d(GpG) *cis*-DDP lesion on strand unwinding catalysed by RecA revealed no effect of the adduct (Fig. 4), in contrast to results for RecB. Several experiments, performed with three different preparations of the partially duplex substrate, gave results comparable to the one shown in Fig. 4. As in the case of RecB, we then investigated the effect of varying concentration of RecA protein on its capacity to unwind un-

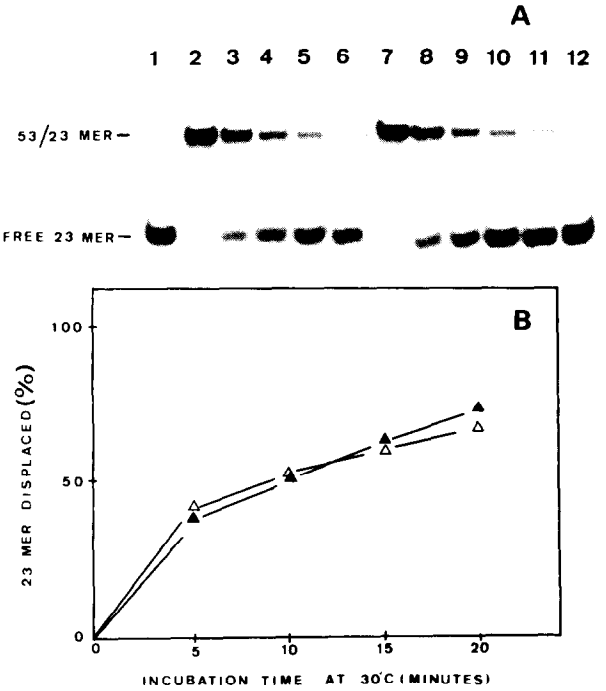


Fig. 4. Effect of d(GpG) *cis*-DDP adduct on the helicase activity of the RecA protein: kinetics of DNA unwinding. Intact or *cis*-DDP treated DNA substrate (110 nM, oligonucleotide) was incubated at 30°C with 750 nM RecA protein in a total volume of 35 μl containing RecA protein buffer and a tenfold excess of unlabeled 23-mer. At the times indicated, 5 μl aliquots were taken and the percentage of 23-mer released determined by polyacrylamide gel electrophoresis as described in section 2. (A) Autoradiogram showing the reaction products: lanes 1 to 6, untreated DNA substrate; lanes 7 to 12, *cis*-DDP treated DNA substrate. Lanes 2 to 6 and 7 to 11: 0, 5, 10, 15 and 20 min time points, respectively. Lanes 1 and 12: heat-denatured substrates. (B) Quantification of the data shown in (A): section corresponding to the radioactive bands shown in Fig. 4(A) were excised from the gel and processed as described in section 2. The amount of the displaced 23-mer was calculated as described in Fig. 2B. Untreated DNA, filled triangles; *cis*-DDP treated DNA, open triangles.

Table II

Turnover number of ATP

	a	b	c
RecB	800 min^{-1}	2870 min^{-1}	1790 min^{-1}
RecA	20 min^{-1}	27 min^{-1}	25.5 min^{-1}

The turnover was measured as described in Section 2 using either 14.2 nM RecB or 530 nM RecA and (a) 600 nM 23-mer alone (b) 600 nM 23-mer plus 60 nM intact duplex 53/23 mers (c) 600 nM 23-mer plus 60 nM duplex 53/23-mers containing the d(GpG) cisplatin adduct. Values represent the average of three independent experiments with RecB and four independent experiments with RecA. No hydrolysis was detected in absence of DNA.

treated or cisplatin treated substrate and found that the d(GpG) lesion did not affect this activity throughout a tenfold range of the protein concentration (Fig. 5).

3.4. *Effect of the d(GpG) adduct on the ATPase activity of the RecB and RecA proteins*

The DNA unwinding activity of both RecB and RecA is dependent upon ATP hydrolysis [16,32]. Therefore, we have compared, under the same experimental conditions used to monitor DNA strand separation, the extent of ATP hydrolysis by RecB and RecA in presence of substrate that was either intact or bearing the d(GpG) *cis*-DDP adduct. As can be seen in Table II, ATP hydrolysis by RecB is significantly reduced in presence of the d(GpG) *cis*-DDP lesion, while RecA ATPase activity remained essentially unchanged (compare columns b and c). Two comments should be made concerning the data shown in Table II. First, the levels of ATPase activity found, which are very similar to the ones reported in literature for both proteins [16,31], are much higher for RecB than for RecA. Second, this activity is readily detectable when the excess of cold 23-mer, normally used as trap in the unwinding reaction, is present as sole substrate (see column a of Table II). Nevertheless, addition of partially duplex 53/23-mers DNA, at one tenth level of the 23-mer alone, increases the ATP hydrolysis by the RecB enzyme of nearly a factor five, indicating its strong preference for this substrate as ATPase cofactor. This finding is in agreement with previous work, showing a dramatic increase in RecB catalysed ATP hydrolysis as a function of DNA length (see Fig. 6 of ref. [21]). In summary, the ATPase data presented indicate that ATP hydrolysis by RecB in presence of a substrate containing a d(GpG) adduct was reduced by about 40% when compared to hydrolysis in the presence of the same amount of intact substrate. This reduction is consistent with the unwinding activity of the protein measured under the same experimental conditions.

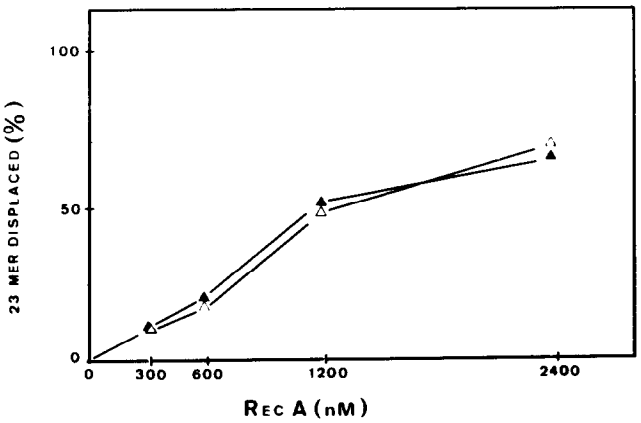


Fig. 5. Effect of the d(GpG) *cis*-DDP adduct on the helicase activity of the RecA protein: protein titration. Intact or *cis*-DDP treated DNA substrate (160 nM, oligonucleotide) was incubated at 30°C with given concentrations of RecA protein buffer and ten-fold excess of unlabeled 23-mer. After a 10 min incubation, the reactions were stopped and the percentage of displaced 23-mer determined as described in the legend to Fig. 2. Untreated DNA, filled triangles; *cis*-DDP treated DNA, open triangles.

4. DISCUSSION

Drug induced damage in DNA has been shown to severely inhibit the action of several enzymes involved in DNA metabolism and the toxicity and mutagenicity of a given lesion could be modulated by the extent of such inhibition. The antitumoral drug *cis*-diamminedichloroplatinum(II), after initial binding to a guanine reacts with another purine base to form bifunctionnal intra and interstrand lesions [33,34]. Over the past years, a number of laboratories have studied the effect of *cis*-DDP lesions on DNA replication and transcription. Some of those studies have been performed on templates containing different types of intrastrand adducts, such as those implicating d(GpG), d(ApG) or d(GpXpG) residues [35,36]. In other instances, substrates containing well defined adducts were used to explore the toxic and mutagenic potential of a given lesion. Along these lines, the role in DNA replication, transcription and repair of the most abundant cisplatin lesion, the d(GpG) adduct, has been recently investigated [4,6,7,9]. In addition to DNA and RNA polymerases, DNA lesions might affect other key enzymes of the DNA metabolism, such as DNA helicases, which play a fundamental role not only in DNA replication and transcription, but also in DNA repair and recombination [28]. Indeed, the activity of helicases associated either with the replicative *E. coli* protein Rep and to the T4 phage replicative protein Dda or with the *E. coli* repair UvrD, have been shown to be inhibited in vitro by the antitumor agent CC-1065 [29,30]. In addition, it has been recently reported that the strand displacement activities of the Rad3 and helicaseII proteins, which are absolutely required for yeast and *E. coli* DNA excision repair, are inhibited by the whole *cis*-DDP intrastrand lesions, although to a different extent [37,38].

We have investigated here the effect of the d(GpG) adduct on the strand separating activity associated with

RecB and RecA proteins, two essential constituents of the process of homologous recombination in *E. coli*.

The RecB protein is a subunit of the RecBCD holoenzyme, a multisubunit complex that is involved in pathways of homologous recombination and DNA repair. The RecBCD holoenzyme can generate single-stranded regions of DNA by virtue of its helicase activity. These single-stranded DNA tails are then used by RecA protein to promote homologous pairing between complementary strands and strand-exchange [27]. Of RecBCD enzyme subunits, only the RecB protein possesses DNA-dependent ATPase and helicase activities [16]. The RecA protein also possesses the ability to unwind short regions of duplex DNA in an ATP-dependent manner. It has been postulated that this activity is required to disrupt regions of secondary structure in single-stranded stretches of DNA, to facilitate the formation of heteroduplex DNA during strand exchange [32,39].

In vitro, most helicases require single-stranded DNA loading sites [28]. For this reason, we constructed the partial DNA duplex depicted in Fig. 1. This DNA substrate contains 23 base pairs of duplex DNA with both 5′ and 3′ single-stranded DNA tails, as well as single, specific d(GpG) *cis*-DDP lesion. Both the RecB and RecA proteins could efficiently displace the 23-mer when annealed to the untreated 53-mer but, while RecA protein could also displace the 23-mer when annealed to the 53-mer containing the d(GpG) *cis*-DDP intrastrand crosslink, the RecB protein appeared to be inhibited by this lesion (see Figs. 2 to 5). This inhibition was consistently observed thoughout a serie of kinetic experiments and by varying the concentrations of the protein in the assay. We estimated that the presence of the *cis*-DDP adduct blocked 40% of the unwinding capacity of RecB. This degree of inhibition can be compared to the one observed for yeast Rad3 and *E. coli* helicase II [38]. In fact, the authors calculated that it required one cisplatin adduct/125 bases in the case of Rad3 protein and one adduct/31 bases in the case of DNA helicaseII to reduce DNA helicase activity to 37%.

Both RecB and RecA require DNA-dependent ATP hydrolysis in order to express their helicase activity [16,32]. We therefore measured ATP hydrolysis during the in vitro strand displacement reaction by these proteins in the same experimental conditions as the gel experiments. As shown in Table II, ATPase activity of RecB appeared to be much greater than RecA. Furthermore, ATP hydrolysis by RecB but not RecA decreased during its attempt to unwind the platinated substrate. ATP hydrolysis and strand separating activity of RecB protein were inhibited by about 40% in the presence of platinated substrate.

Although both proteins could displace a substantial part of the short duplex from the substrate, RecB was more efficient than RecA. Since the reactions reported here were performed in presence of an excess of cold 23-mer, which was used as trap to prevent reannealing of the displaced strand, we asked the question of whether the relative inefficiency of RecA could be attributed to an increased, unproductive binding of RecA to the 23-mer which would sequester part of the protein. To answer this question we carried out a gel retardation experiment [40] using RecB and RecA proteins and 0.6 μM of labeled 23-mer as sole substrate. Even at concentrations of 0.1 μM for RecB and 2 μM for RecA no retardation effect was observed. Under the same experimental conditions, 2 μM *E. coli* single stranded binding protein (SSB) provoked a dramatic shift of the 23-mer (data not shown). Hence, the excess 23-mer did not seem to unproductively bind RecA, and the difference observed in the unwinding and ATPase activities might reflect some intrinsec properties of the proteins or their differential binding to the partially duplex substrate. In this respect, it is interesting to note that RecA forms filaments along single-stranded DNA [31].

What could then be the mechanism of RecB inhibition by the d(GpG) *cis*-DDP adduct? In the case of the yeast Rad3 helicase it had been shown that a variety of DNA lesions, including cisplatin intrastrand adducts, formed a stable protein-damaged DNA complex, as a consequence of which the protein remained sequestered at the lesion encountered during its translocation along the substrate [37]. The same qualitative effect has been observed with helicaseII, although the extent of sequestration is much less pronounced [38]. To test if this hypothesis can be extented to the unwinding activity associated to RecB, we performed four experiments in which the protein was allowed to act on either intact or damaged substrates for times varying from 10 to 20 minutes, then intact 'reporter' substrate was added and the capacity of the protein to displace the newly added substrate was kinetically mesured. We observed no significant differences in the capacity of RecB, 'preincubated' with substrates intact or containing the d(GpG) *cis*-DDP adduct, to unwind the reporter substrate (data not shown). Consequently, the RecB protein could freely dissociate from either DNA substrate to initiate DNA unwinding on the challenge DNA. Thus, it is possible that the RecB protein simply stalls at the lesion as it translocates along the DNA and then dissociates from the substrate. Alternatively, considering that the d(GpG) *cis*-DDP cross link induces a high degree of DNA bending [41,42], the inhibition of the RecB protein-mediated DNA unwinding may be due to the reduced capacity for it to interact with curved DNA.

In conclusion, we have presented evidence that the d(GpG) *cis*-DDP adduct significantly inhibits the DNA helicase and DNA-dependent ATPase activities of the RecB protein, while it had no effect on the same activities associated with the RecA protein. The insensitivity of the RecA protein associated DNA unwinding activity towards this lesion is perhaps no surprise, since it is known that RecA protein promoted strand exchange

can bypass lesions in the DNA including thymine dimers, as well as mismatches [14]. It will now be interesting to see if the d(GpG) *cis*-DDP adduct also significantly inhibits the helicase activity of the RecBCD holoenzyme, since association of the RecB protein with the other subunits results in a complex possessing a highly processive DNA helicase coupled to exo- and endonuclease activities [43]. The RecBCD complex pauses and nicks at a specific DNA sequence, known as the recombinational hotspot *Chi* [27, 44]. It has been proposed that *Chi*-specific cleavage is a result of non-specific nuclease activity of the enzyme that has been sequestered at the site and that any pause of the RecBCD enzyme during its translocation on DNA increases the probability of a nucleolitic event [27 and references therein]. If this hypothesis is correct, inhibition of RecBCD helicase activity by cisplatin, as suggested by our results with RecB, could lead to unscheduled DNA cleavage. Further experiments are needed to test this putative genotoxic effect of cisplatin on homologous recombination.

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