Probing of B—Z Junctions in Recombinant Plasmids in Vitro and in the Cell with Different Osmium Textroxide Complexes

P. BOUBLÍKOVÁ and E. PALEČEK

Institute of Biophysics, Czechoslovak Academy of Sciences, 612 65 Brno, Královopolská 135, Czechoslovakia

Abstract. Complexes of OsO_4 with 2,2′-bipyridine (Os,2,2′-bipy), 4,4′-bipyridine (Os,4,4′-bipy), 1,10-phenanthroline (Os,phe), bathophenanthroline disulfonic acid (Os,bpds) and OsO_4, pyridine reagent (Os,py) were used to probe structural distortions at the junctions between right-handed B and left-handed Z DNA in supercoiled plasmids pRW751 and pPK1 (both containing $(dC—dG)_{13}$ and $(dC—dG)_{16}$ segments). With all five complexes the site-specific modification at the B—Z junctions was detected *in vitro* but only Os, 2,2′-bipy and Os,bpds produced strong site specific modification at submillimolar concentrations. In addition to the B—Z junctions, Os,phe also reacted at other sites. With the exception of Os,2,2′-bipy no one of the tested OsO_4 complexes has proved to be suitable for probing structural distortions at the B—Z junctions in *E. coli* cells.

Key words: B—Z junctions — Supercoiled recombinant plasmids — Osmium tetroxide ligands — DNA structure chemical probing — DNA structure in the cell

Introduction

Osmium tetroxide, pyridine (Os,py) was introduced as a probe of the DNA structure in the beginning of the 80s (Paleček et al. 1981; Lukášová et al. 1982; Paleček and Hung 1983; Glikin et al. 1984; Lukášová et al. 1984). In recent years it has become one of the most frequently used chemical probes of the DNA structure (Glikin et al. 1984; Lilley and Hallam 1984; Lilley and Paleček 1984; Greaves et al. 1985; Johnston and Rich 1985; Lilley et al. 1985; Nejedlý et al. 1985; Vojtíšková et al. 1985; Galazka et al. 1986; McClellan et al. 1986; Galazka et al. 1987; Hanvey et al. 1987; Johnston and Rich 1987; Paleček et al. 1987a, b; Vojtíšková and Paleček 1987; Johnston 1988; Paleček et al. 1988a). It has been applied mainly to studies of local DNA structures in supercoiled plasmids, including cruciforms (Glikin et al. 1984; Lilley and Hallam 1984;

Lilley and Paleček 1984; Lilley et al. 1985; Vojtíšková et al. 1985), B—Z junctions (Johnston and Rich 1985; Nejedlý et al. 1985; Galazka et al. 1986, 1987; Paleček et al. 1987a, b; Johnston 1988), unusual structures at $(dA—dT)_n$ sequences (Greaves et al. 1985; McClellan et al. 1986), curvature-inducing sequences (Paleček et al. 1988a) and homopurine. homopyrimidine sequences (Hanvey et al. 1987; Johnston and Rich 1987; Vojtíšková and Paleček 1987). Even more recently we could show (Paleček et al. 1987c, d, 1988b) that osmium tetroxide with 2,2′-bipyridine (Os,2,2′-bipy) can be applied to studies of the DNA structure in bacterial cells. Using this probe we detected structural distortions at the B—Z junction and offered direct evidence of the existence of left-handed DNA in *E. coli* cells (Paleček et al. 1987c, d, 1988b). In addition we demonstrated the presence of an unusual structure in $(dA—dT)_{16}$ segments of a pAT32 plasmid in the cells (Paleček et al. 1988b).

Our results (Paleček et al. 1987a, c; Vojtíšková and Paleček 1987; Paleček et al. 1988b) have thus shown that the possibility of application of osmium tetroxide as a probe of DNA structure can be significantly extended by using a ligand other than pyridine. It is known that a number of different tertiary amines can be used as ligands to obtain osmium (VI) esters (Schröder 1980). We therefore decided to test some of them differing in size, stereochemistry, electrical charge, etc.

In this paper we used, in addition to pyridine (py) and 2,2′-bipyridine (2,2′-bipy), the following osmium tetroxide ligands (Fig. 1*c*): 4,4′-bipyridine (4,4′-bipy), 1,10-phenanthroline (phe) and bathophenanthroline disulfonic acid (bpds) and tested the ability of the resulting osmium complexes to recognize structural distortions at the B—Z junctions of supercoiled plasmids *in vitro* and *in situ*. Our results show that osmium tetroxide complexes with phe and bpds are able to recognize B—Z junctions *in vitro*. Application of the phe is, however, limited to a narrow range of reaction conditions, as its osmium complex binds under reaction conditions exceeding the defined range. No one of the newly tested osmium complexes was able to recognize the B—Z junction *in situ*.

Materials and Methods

Plasmid DNA. Plasmids pRW751 (Fig. 1*a*) and pK1 (Fig. 1*b*) were isolated from *E. coli* cells, strain JRS 856, by the method described by Holmes and Quigley (1981); the samples were twice deproteinated using chloroform; the final purification step in the case of pRW751 was centrifugation

Abbreviations: py — pyridine; 2,2′-bipy — 2,2′-bipyridine; 4,4′-bipy — 4,4′-bipyridine; phe — phenanthroline (1,10-phenathroline); bpds — bathophenanthroline disulfonic acid, disodium salt; sc-DNA — supercoiled DNA; lin-DNA — linear DNA; rel-DNA — relaxed DNA; EtBr — ethidiumbromide; σ — superhelical density

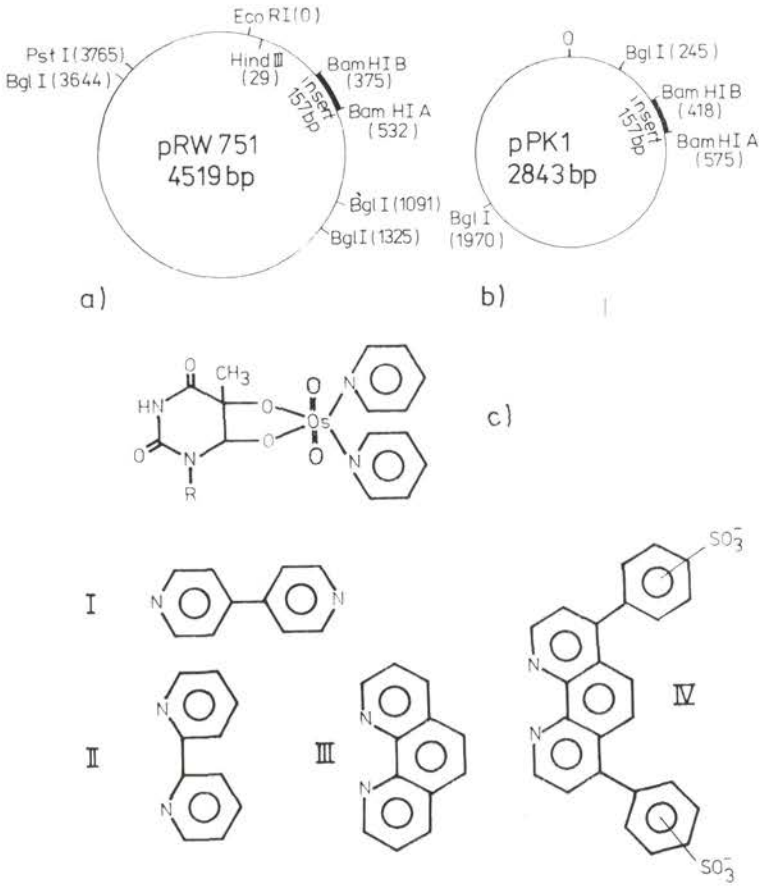

Fig. 1. a, b. Map of plasmids (*a*) pRW751 and (*b*) pPK1. Plasmids were constructed (Kłysik et al. 1982; Paleček et al. 1987c) by cloning the 157 bp insert into the BamHI site of pBR322 and pUC19, respectively; this was done in such a way that the BamHI recognition sequence GGATCC was regenerated at both ends of the insert. Thus BamHI recognition sites in pRW751 and pPK1 lie on the boundary between the $(dC—dG)_n$ blocks and the pBR322 or pUC19 sequence (the first guanine of the recognition sequence being the last guanine of the $(dC—dG)_{13}$ block and the first cytosine of the $(dC—dG)_{16}$ block being the last cytosine of the recognition sequence). The segments $(dC—dG)_{16}$ and $(dC—dG)_{13}$ at the ends of the insert may (given sufficiently negative superhelical density of the plasmids (Singleton et al. 1983; Azorin et al. 1984)) be in the form of left-handed Z-DNA; the plasmids then contain four B—Z junctions, the two "outer" of which reach the area of the restriction sequences B and A of restriction endonuclease BamHI. For more detailed map of 157 bp insert in pRW751 and pPK1 see Kłysik et al. (1982), Nejedlý et al. (1985), Paleček et al. (1987c). *c*) The Os,py complex with thymine and some ligands (I = 4,4′-bipyridine, II = 2,2′-bipyridine, III = 1,10-phenanthroline, IV = bathophenanthroline disulfonic acid) which can replace pyridine in the osmium complexes. The Os,py and Os,2,2′-bipy complexes react with thymine and cytosine residues in DNA (Chang et al. 1977); in the complexes Os,4,4′-bipy, Os,phe and Os,bpds the specificity of the reaction towards bases is not yet known.

in a CsCl/EtBr gradient. The methods of cultivation of *E. coli* cells and amplification of plasmids were described in our previous work (Paleček et al. 1987c).

Chemicals. OsO_4 was obtained rom Fisher Scientific Co., pyridine from Merck, other ligands (2,2'-bipy, 4,4'-bipy, phe, bpds; Fig. 1*c*) from Lachema (ČSSR), all of p.a. purity. Other chemicals were of analytical grade. Restriction endonucleases were obtained from the Institute of Sera and Vaccines (Prague), nuclease S1 was isolated from "Takadiastase" (Vogt 1973).

Modification of plasmid in vitro. The typical reaction mixture contained DNA at a concentration of 40–80 μg/ml in a medium of 25 mmol/l Tris-Cl (pH 7.5), 2.5 mmol/l EDTA, 200 mmol/l NaCl, a maximum of 2 mmol/l OsO_4 plus 2% or 2 mmol/l concentration of other ligand (Fig. 1*c*); pRW751 was modified for 30 min at 26 °C, pPK1 for 20 min at 37 °C. DNA was precipitated with ethanol, extracted with ether and after vacuum drying dissolved in a defined volume of distilled water (Nejedlý et al. 1985; Galazka et al. 1986, 1987; Paleček et al. 1987a, b, c, 1988b).

Modification of plasmid pPK1 in situ. Plasmid-bearing *E. coli* cells were incubated in 2 mmol/l OsO_4 plus 2% py (or at 2 mmol/l concentration of other ligand (Fig. 1*c*)) and 0.5 mol/l potassium phosphate (pH 7.4) for 20 min at 37 °C. The cell concentration in the reaction mixture was about 2 mg dry weight of cells per ml of medium. Modification was stopped by dilution of the sample with a tenfold volume of cooled phosphate buffer (0.1 mol/l, pH 7.4), the cells were centrifuged, rinsed in the same buffer, and kept maximally until the following day, when the modified plasmid was isolated from them (Paleček et al. 1987c, 1988b).

Enzyme cleavage. The modified DNA was cleaved after purification by restriction endonucleases (concentration of restriction endonucleases 1–2 U/μg DNA modified *in vitro*, at least 10 U/μg DNA modified *in situ*). DNA was usually further cleaved with restriction endonuclease BamHI (at least 10 U/μg DNA modified *in vitro*, 20 U/μg DNA modified *in situ*) (pRW751, pPK1), or was precipitated with ethanol and cleaved with S1 nuclease (pRW751).

Electrophoresis. Agarose (Serva) gel (1.5%) electrophoresis was performed using 40 mmol/l Tris-acetate buffer (pH 8.0), 2 mmol/l EDTA at ambient temperature. Gels were stained in ethidium bromide and photographed in midrange UV light (302 nm).

Results

We showed in our previous works (Nejedlý et al. 1985; Paleček et al. 1987a, b) that Os,py and Os,2,2'-bipy specifically modify distorted regions of the B—Z junctions in topoisomeric samples of pRW751. In this work we used pRW751 with native superhelical density (σ), and we obtained results similar to those reported in our previous works (Paleček et al. 1987a, b) with σ more negative than about −0.05, i.e. site-selective modification of all four B—Z junctions of two $(dC—dG)_n$ segments at the ends of the 157 bp insert (Fig. 1*a*; Table 1). The modification of "inner" junctions was detected by nuclease S1 cleavage and that of the "outer" junctions by inhibition of BamHI cleavage (not shown) (BamHI restriction sequences are on the "outer" junctions of pRW751 (Fig. 1*a*)). To

Table 1. Fragments arising after osmium modification and cleavage of plasmids containing B—Z junctions (cf. Fig. 1)

Plasmid	Modification	Cleavage	Fragments
pRW751	—	BamHI	4362 bp, 157 bp
	RS B	BamHI	lin-DNA (4519 bp)
	RS A	BamHI	lin DNA (4519 bp)
	RS B + A	BamHI	sc-DNA
	—	PstI/BamHI	3233 bp, 1129 bp, 157 bp
	RS B	PstI/BamHI	3233 bp, 1286 bp
	RS A	PstI/BamHI	3390 bp, 1129 bp
	RS B + A	PstI/BamHI	lin-DNA (4519 bp)
	—	PstI/S1	lin-DNA (4519 bp)
	B—Z in B	PstI/S1	3259 bp, 1260 bp
	B—Z in A	PstI/S1	3258 bp, 11161 bp
	B—Z in B + A	PstI/S1	3259 bp, 11161 bp, 99 bp
	B—Z out B	PstI/S1	3233 bp, 1286 bp
	B—Z out A	PstI/S1	3390 bp, 1129 bp
	B—Z out B + A	PstI/S1	3233 bp, 1129 bp, 157 bp
pPK1	—	BglI/BamHI	1118 bp, 1395 bp, 173 bp, 157 bp
	RS B	BglI/BamHI	1118 bp, 1395 bp, 330 bp
	RS A	BglI/BamHI	1118 bp, 1552 bp, 173 bp
	RS B + A	BglI/BamHI	1118 bp, 1725 bp

Notes:
— length of fragments arising from cleavage with S1 nuclease is stated only approximately
— short fragments (< 700 bp) were not observed under the selected conditions of electrophoresis (on agarose gels)

RS . . . . . BamHI restriction sequence (cf. Fig. 1)
B . . . . . RS adjacent to $(dC—dG)_{16}$ segment
A . . . . . RS adjacent to $(dC—dG)_{13}$ segment

B—Z in . . . . "inner" B—Z junction (cf. Fig. 1)
B—Z out . . . "outer" B—Z junction (cf. Fig. 1)

B—Z in B . . . . . "inner" B—Z junction proximal to RS B (segment $(dC—dG)_{16}$)
B—Z in A . . . . "inner" B—Z junction proximal to RS A (segment $(dC—dG)_{13}$)
B—Z out B . . . . "outer" B—Z junction involving RS B (segment $(dC—dG)_{16}$)
B—Z out A . . . . "outer" B—Z junction involving RS A (segment $(dC—dG)_{13}$)

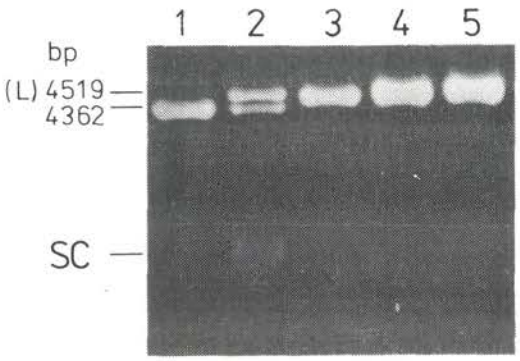

Fig. 2. The effect of Os,bpds modification of pRW751 *in vitro* on cleavage with various restriction endonucleases. Supercoiled DNA was reacted with 2 mmol/l Os,bpds (30 min at 26 °C) and digested with BamHI, EcoRI, Hind III and PstI, respectively (lanes 2—5); for comparison, lane 1 contains unmodified DNA cleaved with BamHI. Positions of linear DNA (L, 4519 bp), supercoiled DNA (SC) and 4362 bp fragment are denoted.

obtain this modification it was necessary to use Os,py at least at a concentration of 1 mmol/l OsO_4 with 1 % py (30 min at 26 °C) while only 0.5 mmol/l OsO_4 with 0.5 mmol/l 2,2′-bipy was sufficient to obtain approximately the same effect.

Os,bpds site-specifically modify B—Z junctions in vitro

Chang et al. (1977) showed that Os,bpds (Fig. 1*c*) reacts with DNA similarly as does Os,py. We were interested whether Os,bpds (with a negative charge and a substantially larger molecule than Os,py) can be used as a probe of DNA structure and selectively modify B—Z junctions in pRW751 at native σ. Treatment of this plasmid with 2 mmol/l Os,bpds resulted in a partial inhibition of BamHI cleavage (Fig. 2, lane 2) while the unmodified control was completely cleaved by BamHI (Fig. 2, lane 1). Restriction endonucleases EcoRI, HindIII and PstI (with a single restriction sequence per molecule distant from the B—Z junction) completely cleaved the modified sample (Fig. 2, lanes 3—5), as did BglI (3 restriction sequences) and BspRI (22 restriction sequences per molecule) (not shown). Treatment of PstI linearized pRW751 with 2 mmol/l Os,bpds (not shown) did not result in any inhibition of BamHI (in agreement with absence of Z DNA in the linearized plasmid).

Site-specific modification of superhelical pRW751 with 2 mmol/l Os,bpds was also recognized by site-specific nuclease S1 cleavage (after linearization with PstI) resulting in an appearance of pairs of fragments (about 3259 bp plus 3358 bp and 1161 plus 1260 bp; Fig. 3*a*, lane 3), corresponding to cleavage at one or both "inner" B—Z junctions. Treatment of the same sample with BamHI

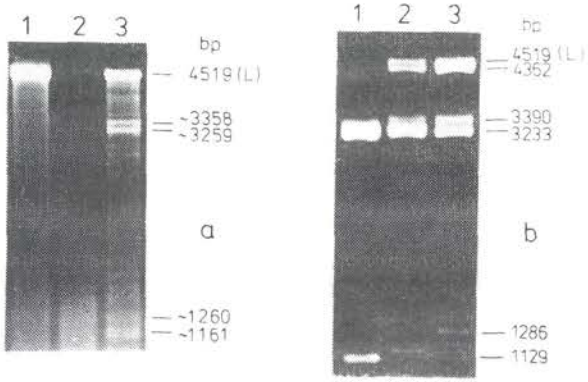

Fig. 3. The effect of Os,phe and Os,bpds modification of pRW751 *in vitro* on cleavage with restriction endonuclease BamHI and nuclease S1. Supercoiled DNA was reacted (30 min at 26 °C) with 2 mmol/l Os,phe (lanes 2) or 2 mmol/l Os,bpds (lanes 3), linearized with PstI and cleaved by a) nuclease S1 or b) BamHI. For comparison, lane 1 contain unmodified DNA cleaved with S1 and/or BamHI. Position of linear DNA (L, 4519 bp) and fragment lengths are denoted.

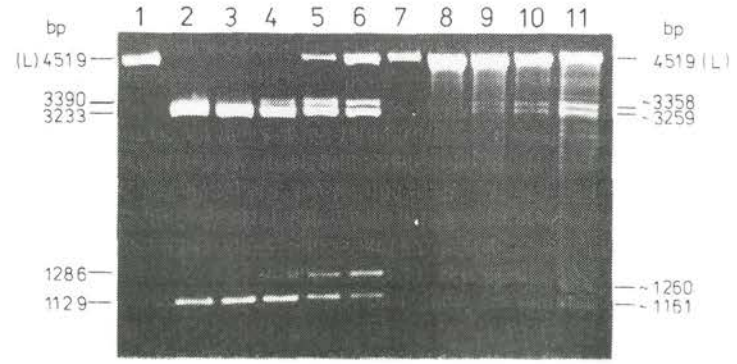

Fig. 4. The dependence of modification of pRW751 *in vitro* on Os,bpds concentration. Supercoiled DNA was reacted (30 min at 26 °C) with 0.2 mmol/l (lanes 3, 8), 0.5 mmol/l (lanes 4, 9), 1.0 mmol/l (lanes 5, 10) or 2.0 mmol/l (lanes 6, 11) Os,bpds, linearized with PstI and cleaved with etheir BamHI (lanes 3—6) or nuclease S1 (lanes 8—11). For comparison, lanes 1, 2, 7 contain unmodified DNA cleaved with PstI, PstI plus BamHI and PstI plus S1, respectively. Position of linear DNA (L, 4519 bp) and fragment lengths are denoted.

produced in addition to fragments corresponding to complete cleavage (3233 bp and 1129 bp), also 3390 bp and 1286 bp fragments, indicating inhibition of BamHI at the "outer" B—Z junctions (Fig. 3*b*, lane 3).

0.5 mmol/l Os,bpds was sufficient to induce site-specific modification at the

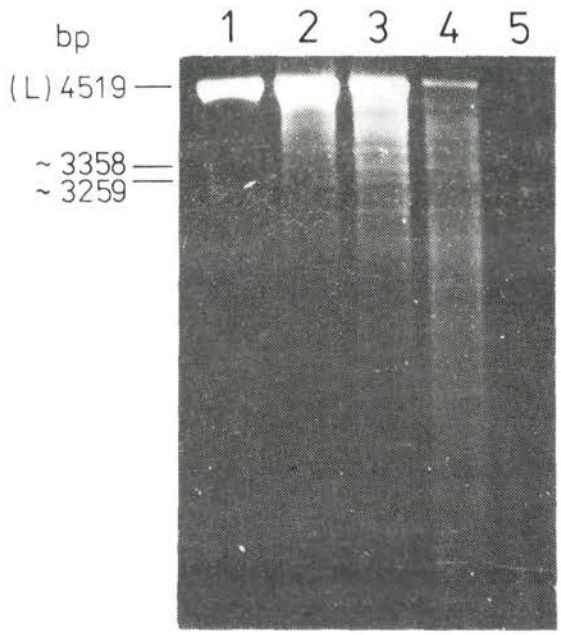

Fig. 5. The dependence of modification of pRW751 *in vitro* on Os,phe concentration. Supercoiled DNA was reacted (30 min at 26 °C) with 0.2 mmol/l (lane 2), 0.5 mmol/l (lane 3), 1 mmol/l (lane 4) or 2 mmol/l (lane 5) Os,phe, linearized with PstI and cleaved with nuclease S1. For comparison, lane 1 contains unmodified DNA cleaved with PstI plus S1. Position of linear DNA (L, 4519 bp) and fragment lengths are denoted.

B—Z junction detectable by nuclease S1 (Fig. 4, lane 9) and inhibition of BamHI cleavage (Fig. 4, lane 4): with growing concentration of Os,bpds intensity of bands corresponding to the specific fragments increased (Fig. 4, lanes 5, 6, 10, 11).

Os,phe reacts at B—Z junctions and at other sites

To our knowledge, Os,phe (Fig. 1*c*) has not been used so far in reaction with DNA. In samples modified with 2 mmol/l Os,phe (30 min at 26 °C) in addition to BamHI (Fig. 3*b*, lane 2), also EcoRI, HindIII, PstI, BglI and BspRI were inhibited (not shown). In agreement with these results cleavage of the osmium modified sample with nuclease S1 (after linearisation with PstI) gave only a number of short fragments (Fig. 3*a*, lane 2) suggesting that the chemical modification occurred at multiple sites. As shown in Fig. 5, with falling Os,phe concentration in the reaction mixture the number of short fragments decreased

and longer fragments appeared. At lower Os,phe concentrations (0.2 to 1.0 mmol/l) very weak bands whose length corresponded to those obtained with other osmium complexes (due to the cleavage at the B—Z junctions) were observed (Fig. 5, lanes 2—4). These results suggest that unlike Os,py, Os,2,2′-bipy and Os,bpds, Os,phe may show some selectivity for the structural distortions at the B—Z junctions only under the conditions inducing small extent of DNA modification while at higher degree of Os,phe modification binding to other sites prevails. In contrast to the results obtained so far with other osmium modified DNAs the electrophoretic mobility of Os,phe modified restriction fragments was altered (Fig. 3*b*, lane 2; Table 1). The mobility changes depended on the extent of modification and on the number of bp contained in the fragments. More details concerning Os,phe reactions with DNA will be published elsewhere.

Os,2,2′-bipy is suited to probe DNA structure in the cell, but Os,phe and Os,bpds are not

We recently showed (Paleček et al. 1987c, d, 1988b) that Os,2,2′-bipy binds at the B—Z junctions of plasmids contained in *E. coli* cells. Here we tried to ascertain whether other OsO_4 complexes behave similarly. We chose a strain of *E. coli* carrying the plasmid pPK1, which we had previously (Paleček et al. 1987c) used in experiments to detect Z DNA in the cell by means of Os,2,2′-bipy. The isolated pPK1 DNA and pPK1-carrying *E. coli* cells were incubated under similar conditions (20 min at 37 °C) in the presence of 2 mmol/l OsO_4 and 2 mmol/l ligands, except for py, which was at 2 % concentration.

pPK1 modified *in vitro* by Os,py, Os,2,2′-bipy and Os,bpds gave on cleavage with BglI/BamHI, apart from 1118 bp and 1396 bp fragments (corresponding to complete BglI/BamHI cleavage (Fig. 6*a*, lane 2)), 1552 bp or 1725 fragments (Fig. 6*a*, lanes 3, 4, 7). These fragments indicate inhibition of BamHI in the restriction sequence A or in both restriction sequences (Fig. 1*b*, Table 1). The sample modified with Os,4,4′-bipy gave only very poor inhibition (BamHI — restriction sequence A) fragment 1552 bp (Fig. 6*a*, lane 5). The sample modified with Os,phe broke into a total of five fragments with electrophoretic mobilities differing from those of the fragments of the other samples (Fig. 6*a*, lane 6).

Plasmid isolated from cells treated with Os,py or Os,2,2′-bipy gave on cleavage with BglI/BamHI a fragment of 1552 bp indicating inhibition of BamHI in the restriction sequence A (Fig. 6*b*, lanes 3, 4). In the case of modification with Os,py cell lysis was observed after only 10 min incubation. Apparently BamHI inhibition also occurred in plasmid isolated from cells treated with Os,phe (Fig. 6*b*, lane 6), similarly as in the case of modification *in vitro* (Fig. 6*a*, lane 6); however, a larger number of fragments was observed (Fig. 6*b*, lane 6)

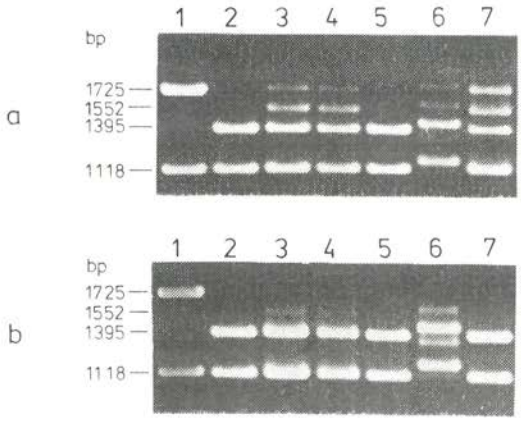

Fig. 6. Modification of DNA plasmid pPK1 with various complexes of OsO_4 *a) in vitro* and *b) in situ*. (*a*) The isolated pPK1 DNA or (*b*) *E. coli* cells carrying pPK1 were incubated (20 min at 37 °C) in a medium containing 2 mmol/l OsO_4 and 2 % py (lane 3) or 2 mmol/l OsO_4 with 2 mmol/l 2,2′-bipy (lane 4), 4,4′-bipy (lane 5), phe (lane 6) or bpds (lane 7). The purified plasmid was cleaved with restriction endonucleases BglI plus BamHI. For comparison, lanes 1 and/or 2 contain unmodified DNA cleaved with BglI and/or BglI plus BamHI. Fragment lengths are denoted.

than in the other modified samples (Fig. 6*b*, lanes 3, 4). Treatment of *E. coli* cells with Os,4,4′-bipy and Os,bpds did not result in inhibition of BamHI (Fig. 6*b*, lanes 5, 7). Our results thus suggest that Os,py, Os,4,4′-bipy, Os,phe and Os,bpds are unsuitable for detection of the structural distortions at the B—Z junctions of pPK1 plasmid in *E. coli* cells.

Discussion

Probing the DNA structure in vitro with various OsO_4 complexes

Due to negative superhelicity local "unusual" structures are stabilized in DNA (Lilley 1984; Gellert and Menzel 1987), and may play an important role in the regulation of gene expression and the realization of further functions of DNA. The use of physical methods to study these local structures is highly limited. On the other hand, chemical probes preferentially reacting with the structurally changed places are highly suitable for this purpose, provided the site of their reaction in the polynucleotide chain can be precisely determined. The first such probes were bromoacetaldehyde and Os,py (Kohwi-Shigematsu et al. 1983; Lilley 1983; Vojtíšková et al. 1983; Paleček 1986). A combination of probes of

known specificity can offer more detailed information on the local structure studied (Johnston and Rich 1985; Paleček 1986; Paleček et al. 1987a, b; Vojtišková and Paleček 1987; Johnston 1988). New chemical probes of DNA structure have thus been sought; e.g. diethyl pyrocarbonate (Herr 1985; Johnston and Rich 1985; Furlong and Lilley 1986; Runkel and Nordheim 1986; Scholten and Nordheim 1986; Johnston 1988) and glyoxal (Lilley et al. 1985; Gough 1988; Lilley 1986; Paleček et al. 1987b; Vojtišková and Paleček 1987) have already proved their worth. OsO_4 can be combined with many ligands affecting the properties of the osmium complexes, which may be of importance for probing of DNA structure.

In this work we investigated three osmium complexes not as yet used as DNA structure probes, Os,phe, Os,bpds and Os,4,4′-bipy (Fig. 1*c*). Os,4,4′-bipy (with 4,4′-bipy at millimolar concentrations) modified the B—Z junctions *in vitro* only very slightly. Similarly as in the case of py, two molecules of 4,4′-bipy form the complex with OsO_4; in view of low solubility of 4,4′-bipy in water it is not possible to attain a concentration comparable to that of py (1—3 %) to obtain sufficient concentration of Os,4,4′-bipy in the modification medium. On the other hand, bidentate Os,bpds offered similar results as did Os,2,2′-bipy when working with DNA *in vitro*. Os,bpds is thus a new probe of DNA structure, usable in submillimolar concentrations (Fig. 4), whose binding site in polynucleotide chain is detectable using S1 nuclease and/or inhibition of restriction cleavage. In experiments *in vitro* Os,bpds can yield the same results as Os,2,2′-bipy, except that the negatively charged bulky complex might appear more selective with tiny conformational changes and in DNA-protein interaction studies.

The results obtained with Os,phe in this work show that the properties of this complex differ greatly from those of osmium probes known to date. Apart from the weak reaction at the B—Z junctions (Fig. 5, lane 2—4), there is also a binding of Os,phe at other sites in the molecule. This binding might be probably connected with intercalation of Os,phe into DNA analogous to the binding of the complex with platinum (Lippard et al. 1976). Further work is required to draw definitive conclusions on the nature of Os,phe binding to DNA. The very fact that Os,phe binds covalently to DNA is interesting, and offers the prospect of making use of this complex in DNA research. phe has in recent years been used in a reaction mixture with other substances to generate $OH^{\bullet}$-radicals, inducing site-specific cleavage of the polynucleotide chain (Barton 1986; Sigman 1986; Chen and Sigman 1987). The combination of findings obtained with this system and with Os,phe could open up new possibilities for the use of phe in studying DNA structure.

Structure of the B—Z junction

The results obtained so far by testing the B—Z junctions in supercoiled plasmids with chemical probes (Johnston and Rich 1985; Nejedlý et al. 1985; Galazka et al. 1986, 1987; Paleček et al. 1987a, b; Johnston 1988) suggest that the B—Z junction is a narrow structurally distorted region whose detailed spatial arrangement may vary in dependence on flanking sequences, superhelical density and other factors. We have shown that the B—Z junctions in pRW751 are site-specifically modified by chloroacetaldehyde (Paleček et al. 1987a) and glyoxal (Paleček et al. 1987b), i.e. agents requiring non-paired bases for the reaction to occur. Similar results have recently been obtained by other authors with bromoacetaldehyde (McLean et al. 1987) and chloroacetaldehyde (Kohwi-Shigematsu et al. 1987). Pőrschke et al. (1987) demonstrated that the hydrodynamic dimensions of the 153-bp fragment (containing two $(dC—dG)_n$ segments) are not affected by the presence of two B—Z junctions. Does this mean that hydrogen bonds are broken only in one base pair, without much influence on base stacking? Such a change would not be expected to alter local flexibility of the molecule, nor to induce a strong curvature of the molecule. On the other hand, the results of probing of the B—Z junctions in supercoiled plasmids with Os,py (Johnston and Rich 1985; Nejedlý et al. 1985; Galazka et al. 1986, 1987; Paleček et al. 1987a, b; Johnston 1988), Os,2,2′-bipy (Paleček et al. 1987a) and glyoxal (Paleček et al. 1987b) suggest that at least one AT and one GC pair are chemically modifiable. The results obtained with bulky Os,bpds (Fig. 4) might also be interpreted in terms of changes involving more than one base pair. For better interpretation of this result model building will, however, be necessary. On the other hand, it cannot be excluded that the B—Z junction in a linear fragment (Z DNA induced by $[Co(NH_3)_6]^{3+}$) (Pőrschke et al. 1987) may structurally differ from that in a supercoiled plasmid. This assumption is supported by the results of chemical probing of the B—Z junctions (Nejedlý et al. 1985; Paleček et al. 1987a, b) at superhelical densites more negative than those necessary for B—Z transition. Experiments to test these assumption are in progress.

Probing of the DNA structure in the cell

Recently we have shown that Os,2,2′-bipy can be applied for the studies of DNA structure *in situ* and offered the first direct and unequivocal evidence of the existence of left-handed DNA in the bacterial cell (Paleček et al. 1987c, d, 1988b). Jaworski et al. (1987), using a special molecular genetic technique, have shown independently that left-handed DNA exists and elicits a biological response in *E. coli* cells. Their evidence lies in the inhibition of methylation of a restriction EcoRI site in the Z DNA segment or at the B—Z junction. Such an

inhibition may be due directly to the presence of left-handed DNA or to the binding of a specific protein which prevents the restriciton site in DNA being methylated. The evidence obtained with a chemical probe is more direct, but this technique can hardly avoid perturbance of the intracellular environment, due to the penetration of Os,2,2′-bipy into the cell. Thus the results obtained with the chemical and molecular genetic techniques complement each other, bringing strong evidence of the existence of left-handed DNA *in vivo*. In contrast to the molecular genetic technique (Jaworski et al. 1987) which was designed especially to study left-handed DNA in the bacterial cell, the chemical probe can be applied to studies of various local structures which contain base accessible to a chemical probe.

In this paper we attempted to find osmium complexes other than Os,2,2′-bipy which may be applied as probes of DNA structure *in situ*. We obtained positive results with Os,py (Fig. 6*b*, lane 3); this agent induced, however, lysis of *E. coli* cells at pyridine concentrations necessary for the reaction. At higher pyridine concentrations lysis proceeded faster. It cannot be excluded that Os,py may serve as a probe of the DNA structure *in situ* with some other cells which resist the relatively high concentration of pyridine. The results obtained with Os,phe (Fig. 6*b*, lane 6) suggest that this agent also is able to penetrate into *E. coli* cells; no cell lysis was observed under the given conditions, but the Os,phe action on DNA is not yet fully understood, and Os,phe cannot thus be considered as a probe of DNA structure at present. The negative result obtained with Os,bpds (Fig. 6*b*, lane 7) can be explained either by inability of this agent to penetrate the cell membrane, or by a sterical hindrance which prevents the bulky, negatively charged Os,bpds from site-specifically reacting at the the B—Z junction in the cell environment. Even if Os,bpds cannot penetrate into the *E. coli* cell, it may find an application as a probe of DNA structure *in situ* with some other cells or viruses. Further work will be necessary to elucidate the potentialities of this probe. Thus, at present Os,2,2′-bipy represents the only chemical probe capable of recognizing "open" DNA structures (Paleček 1976) in the cell. This agent easily penetrates into *E. coli* cells without attacking their integrity (Paleček et al. 1987c). Other aspects of the influence of this agent on the cell are currently being studied.

Acknowledgement. The authors wish to express their gratitude to Professor R. D. Wells and Dr. J. Klysik for their generous gift of the recombinant plasmid pRW751.

References

Azorin F., Hahn R., Rich A. (1984): Restriction endonuclease can be used to study B—Z junctions in supercoiled DNA. Proc. Nat. Acad. Sci. USA **81**, 5714—5718

Barton J. K. (1986): Metals and DNA: Molecular left-handed complements. Science **233**, 727—734

Chang C.-H., Beer M., Marzilli L. G. (1977): The reaction of osmium tetroxide with deoxyribonucleic acid and synthetic polynucleotides in the presence of tertiary nitrogen donor ligands. Biochemistry **16**, 33—38

Chen C.-H., Sigman D. S. (1987): Chemical conversion of a DNA-binding protein into a site-specific nuclease. Science **237**, 1197—1201

Furlong J. C., Lilley D. M. J. (1986): Highly selective chemical modification of cruciform loops by diethyl pyrocarbonate. Nucl. Acid Res. **14**, 3995—4007

Gałazka G., Paleček E., Wells R. D., Kłysik J. (1986): Site-specific OsO_4 modification of the B—Z junction formed at the $(dA—dC)_{12}$ region in supercoiled DNA. J. Biol. Chem. **261**, 7092—7098

Gałazka G., Kłysik J., Kwinkowski M., Szala S., Paleček E. (1987): B—Z transition within the mouse repetitive sequences. In: Biophosphates and Their Analogues — Synthesis, Structure, Metabolism and Activity (Eds. K. S. Bruzik and W. J. Stec), pp. 515—524, Elsevier Science Publishers B. W., Amsterdam

Gellert M., Menzel R. (1987): DNA supercoiling as a regulator of bacterial gene expression. In: New Frontiers in the Study of Gene Functions (Eds. G. Poste and S. T. Crooke), pp. 51—59, Plenum Publishing Corporation

Glikin G. C., Vojtíšková M., Rena-Descalzi L., Paleček E. (1984): Osmium tetroxide: a new probe for site-specific distortions in supercoiled DNAs. Nucl. Acid Res. **12**, 1725—1735

Gough G. W. (1986): Studies of the structure and chemical reactivity of cruciform structures in supercoiled DNA molecules. PhD. Thesis, Univ. Dundee

Greaves D. R., Patient R. K., Lilley D. M. J. (1985): Facile cruciform formation by an $(A—T)_{34}$ sequence from a Xenopus globin gene. J. Mol. Biol. **185**, 461—478

Hanvey J. C., Collier D. A., Griffin J., Kilpatrick M. W., Kłysik J., McLean M. J., Wohlrab F., Wells R. D. (1987): Unusual DNA structures adopted by natural and synthetic pur,pyr sequences in supercoiled plasmids. Fifth Conversation in Biomolecular Stereodynamics, Albany, Book of abstracts, p. 78

Herr W. (1985): Diethyl pyrocarbonate: a chemical probe for secondary structure in negatively supercoiled DNA. Proc. Nat. Acad. Sci. USA **82**, 8009—8013

Holmes D. S., Quigley M. (1981): A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem. **114**, 193—198

Jaworski A., Hsieh W.-T., Blaho J. A., Larson J. E., Wells R. D. (1987): Left-handed DNA in vivo. Science **238**, 773—777

Johnston B. H. (1988): Chemical probing of the B—Z transition in negatively supercoiled DNA. J. Biomol. Struct. Dyn. **6**, 153—166

Johnston B. H., Rich A. (1985): Chemical probes of DNA conformation: Detection of Z-DNA at nucleotide resolution. Cell **42**, 713—724

Johnston B. H., Rich A. (1987): Structural features of Z-DNA and the B—Z transition deduced from chemical probing experiments. Fifth Conversation in Biomolecular Stereodynamics, Albany, Book of abstracts, p. 7

Kłysik J., Stirdivant S. M., Wells R. D. (1982): Left-handed DNA. Cloning, characterization and instability of inserts containing different lengths of (dC—dG) in *Escherichia coli*. J. Biol. Chem. **257**, 10152—10158

Kohwi-Shigematsu T., Gelinas R., Weintraub H. (1983): Detection of an altered DNA confor-

mation at specific sites in chromatin and supercoiled DNA. Proc. Nat. Acad. Sci. USA **80,** 4389—4393

Kohwi-Shigematsu T., Manes T., Kohwi Y. (1987): Unusual conformational effect exerted by Z-DNA upon its neighboring sequences. Proc. Nat. Acad. Sci. USA **84,** 2223—2227

Lilley D. M. J. (1983): Structural perturbation in supercoiled DNA: hypersensitivity to modification by a single-stranded-selective chemical reagent. Nucl. Acid Res. **11,** 3097—3112

Lilley D. M. J. (1984): DNA: Sequence, structure and supercoiling. Biochem. Soc. Trans. **12,** 127—140

Lilley D. M. J. (1986): Cyclic adduct formation at structural perturbations in supercoiled DNA molecules. In: The Role of Cyclic Nucleic Acid in Carcinogenesis and Mutagenesis (Eds. B. Singer and B. H. Brasch), pp. 83—99, IARC Scientific Publications no. 70, Lyon

Lilley D. M. J., Hallam L. R. (1984): Thermodynamics of the ColE1 cruciform. J. Mol. Biol. **189,** 179—200

Lilley D. M. J., Paleček E. (1984): The supercoiled-stabilised cruciform of ColE1 is hyper-reactive to osmium tetroxide. EMBO J. **3,** 1187—1192

Lilley D. M. J., Gough G. W., Hallam L. R., Sullivan K. M. (1985): The physical chemistry of cruciform structures in supercoiled DNA molecules. Biochimie **67,** 697—706

Lippard S. J., Bond P. J., Wu K. C., Bauer W. (1976): Stereochemical requirements for intercalation of platinum complexes into double-stranded DNA's. Science **194,** 726—728

Lukášová E., Jelen F., Paleček E. (1982): Electrochemistry of osmium-nucleic acids complexes: A probe for single-stranded and distorted double-stranded regions in DNA. Gen. Physiol. Biophys. **1,** 53—70

Lukášová E., Vojtíšková M., Jelen F., Sticzay T., Paleček E. (1984): Osmium-induced alteration in DNA structure. Gen. Physiol. Biophys. **3,** 175—191

McClellan J. A., Paleček E., Lilley D. M. J. (1986): $(A—T)_n$ tracts embedded in random sequence DNA — formation of a structure which is chemically reactive and torsionally deformable. Nucl. Acid Res. **14,** 9291—9309

McLean M. J., Larson J. E., Wohlrab F., Wells R. D. (1987): Reaction conditions affect the specifity of bromoacetaldehyde as a probe for DNA cruciforms and B—Z junctions. Nucl. Acid Res. **15,** 6917—6937

Nejedlý K., Kwinkowski M., Gałazka G., Kłysik J., Paleček E. (1985): Recognition of the structural distortions at the junctions between B and Z segments in negatively supercoiled DNA by osmium tetroxide. J. Biomol. Struct. Dyn. **3,** 467—478

Paleček E. (1976): Premelting changes in DNA conformation. In: Progress in Nucleic Acids Research and Molecular Biology (Ed. W. E. Cohn), pp. 151—231, Vol. **18,** Academic Press, New York

Paleček E. (1986): Chemical and electrochemical probes of the DNA polymorphic structure. Stud. Biophys. **114,** 39—48

Paleček E., Hung M. A. (1983): Determination of nanogram quantities of osmium-labeled nucleic acids by stripping (inverse) voltammetry. Anal. Biochem. **132,** 236—242

Paleček E., Lukášová E., Jelen F., Vojtíšková M. (1981): Electrochemical analysis of polynucleotides. Biochem. Bioenerg. **7,** 317—332

Paleček E., Boublíková P., Gałazka G., Kłysik J. (1987a): Inhibition of restriction endonuclease cleavage due to site-specific chemical modification of the B—Z junction in supercoiled DNA. Gen. Physiol. Biophys. **6,** 327—341

Paleček E., Boublíková P., Nejedlý K., Gałazka G., Kłysik J. (1987b): B—Z junctions in supercoiled pRW751 contain unpaired bases or non-Watson-Crick base pairs. J. Biol. Struct. Dyn. **5,** 297—306

Paleček E., Boublíková P., Karlovský P. (1987c): Osmium tetroxide recognized structural distor-

tions at junctions between right- and left-handed DNA in a bacterial cell. Gen. Physiol. Biophys. **6,** 593—608

Paleček E., Boublíková P., Nejedlý K., Vojtíšková M. (1987d): Chemical probes of the DNA polymorphic structure. EMBO Workshop on the Structure and Dynamics of Superhelical DNA, Autrans/Grenoble, Book of Abstracts

Paleček E., Makaturová-Rašovská E., Diekmann S. (1988a): Osmium tetroxide probing of local DNA structure in linear and supercoiled plasmids containing curvature-inducing sequences. Gen. Physiol. Biophys. **7,** 379—393

Paleček E., Rašovská E., Boublíková P. (1988b): Probing of DNA polymorphic structure in the cell with osmium tetroxide. Biochem. Biophys. Res. Commun. **150,** 731—738

Pőrschke D., Zacharias W., Wells R. D. (1987): B—Z junctions are neither highly flexible nor strongly bent. Biopolymers **26,** 1971—1974.

Runkel L., Nordheim A. (1986): Chemical footprinting of the interaction between left-handed DNA and anti-Z-DNA antibodies by diethyl pyrocarbonate carbethoxylation. J. Mol. Biol. **189,** 487—501

Scholten P. M., Nordheim A. (1986): Diethyl pyrocarbonate: a chemical probe for DNA cruciforms. Nucl. Acid Res. **14,** 3981—3993

Schrőder M. (1980): Osmium tetroxide cis hydroxylation of unsaturated substrates. Chem. Rev. **80,** 187—213

Sigman D. S. (1986): Nuclease activity of 1,10-phenanthroline copper ion. Account Chem. Res. **19,** 180—186

Singleton C. K., Kłysik J., Wells R. D. (1983): Conformational flexibility of junctions between contiguous B- and Z-DNA in supercoiled plasmids. Proc. Nat. Acad. Sci. USA **80,** 2447—2451

Vogt V. M. (1973): Purification and further properties of single-strand-specific nuclease from *Aspergillus oryzae*. Eur. J. Biochem. **33,** 192—200

Vojtíšková M., Paleček E. (1987): Unusual protonated structure in the homopurine homopyrimidine tract of supercoiled and linearized plasmids recognized by chemical probes. J. Biomol. Struct. Dyn. **5,** 283—296

Vojtíšková M., Glikin G., Nejedlý K., Paleček E. (1983): Osmium induced alteration in plasmid DNA structure. FEBS 4th Symposium on DNA, Liblice, Abstracts of the Communications, p. 37

Vojtíšková M., Štokrová J., Paleček E. (1985): Modification of ColE1 DNA with osmium tetroxide generates positively supercoiled molecules. J. Biomol. Struct. Dyn. **2,** 1013—1019

Final version accepted April 30, 1989