Osmium Tetroxide Reactivity of DNA Bases in Nucleotide Sequencing and Probing of DNA Structure

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Abstract. Osmium tetroxide, 2,2'-bipyridine (Os,bipy) has been widely applied as a probe of the DNA structure. To obtain information about reactivity of DNA bases toward this probe synthetic homopolynucleotides poly(dT), poly(dC), poly(dG) and poly(dA) were treated with Os,bipy and the content of modified bases measured by stripping voltammetry and absorption spectrophotometry. After 20 hours' treatment strong modification of poly(dT) and poly(dC) and weak modification of poly(dG) were observed, while no modification was detected in poly(dA). At short incubation times under conditions close to those usually used in probing the DNA structure the extent of poly(dT) modification was more than 10 times higher than that of poly(dC). Thus, in single-stranded DNA Os,bipy reacts with T >> C and G. Due to the fast reaction of thymines with Os,bipy (and osmium tetroxide, pyridine) these chemicals can be applied in Maxam-Gilbert nucleotide sequencing as agents specific for thymines in single-stranded DNA.

Key words: Reactivity of DNA bases to osmium tetroxide — DNA nucleotide sequencing — Chemical probing of DNA structure.

Introduction

At the beginning of the 80s we showed (Paleček et al. 1981; Lukášová et al. 1982; 1984; Paleček and Hung 1983) that osmium tetroxide, pyridine (Os,py) reacts preferentially with single-stranded and distorted double-stranded regions in DNA and can be applied as a probe of DNA structure. In recent years Os,py has become one of the most frequent chemical probes used in studies of local structures in supercoiled DNAs (for review see Paleček et al. 1990), including cruciforms (Lilley and Paleček 1984), B-Z junctions (Paleček et al. 1987a; Johnston 1988a; Nejedlý et

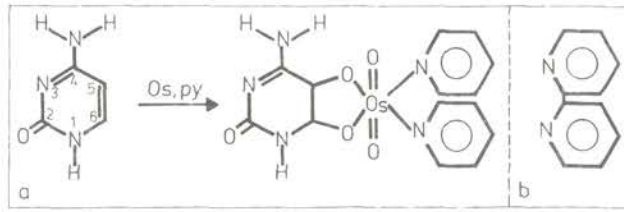

Figure 1. (a) formation of adduct between cytosine, osmium tetroxide and pyridine; (b) bipy.

al. 1985; 1988) unusual structures at $(dA\text{-}dT)_n$ sequences (McClellan et al. 1986; Nejedlý et al. 1989), curvature-inducing sequences (Paleček et al. 1988a) and homopurine.homopyrimidine sequences (Vojtíšková et al. 1987; 1988; Johnston 1988b, Htun and Dahlberg 1988; Hanvey et al. 1988a). Recently we have shown (Paleček et al. 1987b, 1988b, McClellan et al. 1990; Boublíková and Paleček 1990) that osmium tetroxide, 2,2'-bipyridine (Os,bipy) can be used as a probe of DNA structure *in situ*. In spite of its wide application the specificity of Os,py and Os,bipy probes and the chemistry behind it have not yet been well understood.

In 1977, Sverdlov et al. found that osmium tetroxide in 0.1 mol/l NH_3 can be used for the sequencing of thymidine residues in deoxyoligonucleotides. Later Friedmann and Brown (1978) showed that treatment of double-stranded DNA with osmium tetroxide in dilute pyridine followed by treatment with piperidine leads to chain cleavage at the modified thymidine residues.

It has been shown by other authors (Highton et al. 1968; Subbaraman et al. 1971) that osmium tetroxide in the presence of suitable ligands adds to olefins to produce osmate esters with osmium in oxidation state 6+. This reaction depends on the chemical nature of the olefin and on the ligands present in the reaction mixture. In the absence of ligands osmium tetroxide reacts with thymines while the reactions with other DNA bases are negligible (Beer et al. 1966). On the other hand, it has been shown (Chang et al. 1977; Lukášová et al. 1982) that in the presence of pyridine osmium tetroxide not only reacts with thymine, but also with cytosine (Fig. 1). One may ask why Friedmann and Brown (1978) observed the specific chain cleavage only at thymidines and not at deoxycytidines. Was it because they worked with double-stranded DNA in which cytosines were not available for the reaction? Or was it due to a different reactivity of thymidine and cytidine residues?

In a attempt to answer these questions we modified synthetic polydeoxynucleotides and single-stranded DNA under various conditions and determined the extent of their modification. We found that, under certain conditions, Os,py and Os,bipy show high preference for thymines in single-stranded DNA and can be

applied as specific agents for thymidine in nucleotide sequencing. Under stronger reaction conditions the amount of modified cytosine residues may be close to that of thymines.

Materials and Methods

Poly(dC), poly(dA), poly(dG) and poly(dT) were from P-L Biochemicals; additional poly(dT) was kindly donated by Prof. W. Guschlbauer. Poly(rU) and poly(rC) were from Sigma. OsO_4 was purchased from Fischer Scientific Co., 2,2'-bipyridine (bipy) from Lachema, and pyridine from Merck. Other chemicals were of analytical grade. Calf thymus DNA was isolated by the method of Zamenhof (1957). If not stated otherwise, thermal denaturation was performed by heating the DNA samples in 7.5×10^{-3} mol/l NaCl, 7.5×10^{-4} mol/l sodium citrate, pH 7 (SSC/20) (100 °C, 6 min) followed by rapid cooling in an ice bath.

Osmium modification of DNA. A typical reaction mixture contained denatured DNA or synthetic polynucleotides at 1x 10^{-3} mol/l or submillimolar concentrations with Os,bipy at 1.5 to four-fold excess as indicated in the text. The mixture was incubated at 26 °C and the reaction was stopped by extracting the samples twice with chloroform followed by overnight dialysis against SSC/20 at 4 °C; DNA concentration was calculated from the phosphorus content determined by the method of Chen et al. (1956). Alternatively, the reaction mixture was passed through a Sephadex G-25 column NAP-5 (Pharmacia), and the calculations of the DNA concentrations were based on the assumption that practically all DNA was eluted (this procedure was applied only at mild reaction conditions). The content of modified bases in DNA (or RNA) was determined by electrochemical analysis and absorption spectroscopy.

Absorption spectroscopy. It has been shown (Chang et al. 1977) that adducts of Os,bipy with nucleic acid display a characteristic maximum around 310 nm. The molar extinction coefficients at this wavelength (ϵ_{310}) were determined as 11,000 and 6,200 $cm^{-1}.mol^{-1}.l$ for poly(dC) and DNA adducts, respectively. ϵ_{310} for poly(dT) was 13,500 $cm^{-1}.mol^{-1}.l$ both after 20 hour and 45 min modification under conditions given in Tab. I. The values of ϵ_{310} of the synthetic polynucleotides were used to determine the osmium content in poly(dC) and poly(dT) spectrophotometrically and to calibrate the electrochemical method; as no fully modified poly(dG) was available, fully unmodified poly(dT) was used for calibration of the electrochemical determination of poly(dG). Absorption spectroscopic measurements were carried out with Zeiss VSU-2, Specord M 40 or Unicam SP 1700 instruments.

Electrochemical analysis. We have previously shown (Paleček et al. 1981; Lukášová et al. 1982; 1984; Paleček and Hung 1983) that the DNA-Os,py adducts can be determined by means of polarography and voltammetry using the mercury drop electrode. The highest sensitivity of the determination was achieved with differential pulse stripping voltammetry (DPSV) (Paleček and Hung 1983). This technique is based on the accumulation of osmium modified DNA at the electrode with subsequent osmium reduction. In this work we used DPSV for the determination of the DNA-Os,bipy adduct which produced a DPSV peak at a potential of about −1.2 V (vs. Ag/AgCl/sat. KCl electrode) (Fig. 2) corresponding to the peak potential of DNA-Os,py; basically, also other DPSV features of DNA-Os,bipy agreed with those of DNA-Os,py (Paleček and Hung 1983).

If denatured DNA was treated with 5×10^{-4} mol/l Os,bipy for 120 min at 26 °C, a

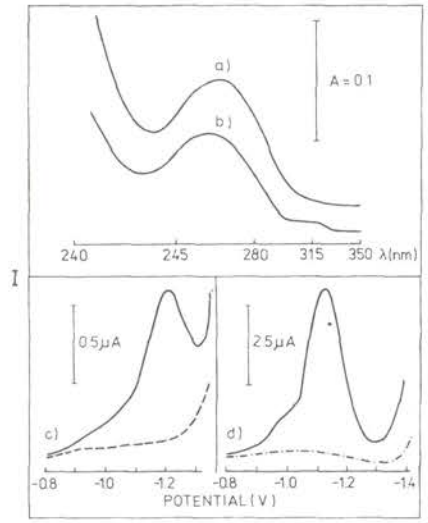

Figure 2. Absorption spectra (a,b) and differential pulse stripping voltammograms (c,d) of DNA modified with Os,bipy. Thermally denatured calf thymus DNA at a concentration of 70 μg/ml in 0.05 mol/l sodium phosphate was treated with 5×10^{-4} mol/l OsO_4,5×10^{-4} mol/l bipy at 26 °C for (a,c) 15 min or (b,d) 120 min, extracted twice with chloroform and dialysed. Before DPSV measurements DNA concentration was adjusted to 0.9 μg/ml. Initial potential −0.8 V, accumulation time 120 s; (– – ––) background electrolyte 0.3 mol/l ammonium formate with 0.05 mol/l sodium phosphate (pH 6.8); (– · – · – · –) unmodified DNA.

well-developed DPSV peak appeared (Fig. 2*d*) and also a distinct band at about 310 nm was observed in the absorption spectra (Fig. 2*b*). DNA treated with Os,bipy for only 15 min under the same conditions produced a DPSV peak characteristic of osmium-modified DNA (Fig. 2*c*) while no band around 310 nm was observed in the absorption spectra. Thus, DPSV is better suited for the detection of small extents of DNA modification with Os,bipy than is absorption spectrophotometry. We therefore applied the former technique especially in those cases where only a small fraction of bases in the DNA molecule was expected to be modified. All measurements were performed at full coverage of the electrode surface to eliminate the influence of possible differences in the diffusion coefficients of the polynucleotides studied. Electrochemical measurements were carried out on a PAR 174A Polarographic Analyser in connection with a model 303A SMDE electrode and Omnigraphic Model 9002A X-Y recorder. A three-electrode system was used, including a hanging mercury drop electrode (HMDE) as the working electrode, Pt wire as the auxiliary electrode, and Ag/AgCl/sat. KCl electrode as the reference electrode. Volume of the analysed samples was usually 1.2 ml. Instrument settings: scan rate 10 mV/s, modulation amplitude 50 mV, pulses applied at 0.5 s intervals, deposition time as required, equlibration time 15 s, stirring 400 rpm, drop size 0.94 mm^2, initial potential −0.8 V.

Table 1. Os/P Ratios of the Adducts of Polynucleotides with OsO_4, 2,2'-bipyridine

Polynucleotide	Reaction time	Os/P	
		DPSV	spectra
Poly(dT)	20 hours	1.00	1.00
	15 min	0.90	-
	45 min	1.00	1.00
Poly(dC)	20 hours	0.85	0.85
	15 min	0.08	=
Poly(dG)	20 hours	0.07	=
	15 min	0.05	=
Poly(dA)	20 hours	0.00	=
	15 min	0.00	=

1×10^{-3} mol/l polynucleotide in 0.05 mol/l sodium phosphate (pH 7.0) was treated with 4×10^{-3} mol/l osmium tetroxide, 4×10^{-3} mol/l bipy at 26 °C for 20 hours (conditions S); for shorter modification times, 2.5×10^{-4} mol/l polynucleotide with 1×10^{-3} mol/l osmium tetroxide, 1×10^{-3} mol/l bipy were used under otherwise the same conditions; -, Os/P not measured; =, no Os detected. For other details see the text.

DNA sequencing. A DNA fragment originating from *Phytophora parasitica* genome was labelled at one terminus with ^{32}P using filling-in reaction of Klenow fragment of DNA polymerase I, precipitated with ethanol and solubilized in water with 1×10^{-3} g/ml sonicated salmon sperm DNA. Standard sequencing reactions were done according to Maxam and Gilbert (1980). Osmium modification: DNA was denatured at 100 °C for 3 min and incubated with 1×10^{-3} mol/l OsO_4 and pyridine (1%) or bipy (1×10^{-3} mol/l) for 4 or 16 min at 24 °C. The reaction was interrupted and DNA precipitated by mixing 5 vols. of 3 mol/l sodium acetate(pH 6.6)-mercaptoethanol-ethanol (3:5:90). Piperidine cleavage was done as described by Maxam and Gilbert (1980).

Results

Os,bipy reacts with T, C and G residues in DNA. To obtain information about reactivity of DNA bases towards Os,bipy we modified synthetic polynucleotides poly(dC), poly(dT), poly(dA) and poly(dG) under strong reaction conditions S (close to those used earlier by Chang et al. (1977) with polyribonucleotides) and under much milder reaction condition M used usually in probing DNA structure (e.g. Lilley and Paleček 1984; Paleček et al. 1987a; Nejedlý et al. 1985; 1988). The extent of modification was determined by differential pulse stripping voltammetry (DPSV) (Paleček and Hung 1983) and from the absorption spectra (Chang et al. 1977).

Strong reaction condition S. 1×10^{-3} mol/l polynucleotide was treated with 4×10^{-3}

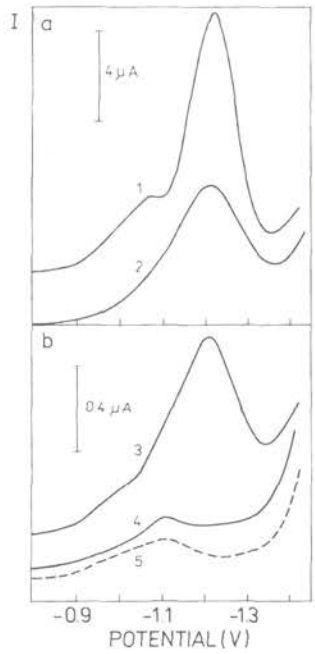

Figure 3. DP stripping voltammograms of four polydeoxynucleotides treated with Os,bipy under strong reaction conditions S. (a) 1, poly(dT); 2, poly(dC); (b) 3, poly(dG); 4, poly(dA); 5, (– – – –) background electrolyte. Polynucleotides at 1×10^{-3} mol/l concentration (related to monomer content) were treated with 4×10^{-3} mol/l osmium tetroxide, 4×10^{-3} mol/l bipy for 20 hours at 26 °C in 3.5×10^{-2} mol/l sodium phosphate (pH 7.0) and dialysed. DPSV measurements were performed at a polynucleotide concentration of 0.6 μg/ml; for other conditions see legend to Fig. 2.

mol/l osmium tetroxide, 4×10^{-3} mol/l bipy at 26 °C for 20 hours. After this treatment both pyrimidine bases containing polynucleotides were strongly modified (Tab. I, Fig. 3). In poly(dG) the Os/P ratio was 0.07 and in poly(dA) the extent of modification was below the detection limit.

Mild reaction conditions M. 2.5×10^{-4} mol/l polynucleotides were treated with 1×10^{-3} mol/l osmium tetroxide, 1×10^{-3} mol/l bipy at 26 °C for 15 min. Due to this treatment, 90% of bases in poly(dT) were modified (Tab. I) while modification of poly(dC) corresponded only to about 8%; 2 hours' treatment at smaller Os,bipy excess (Fig. 4) yielded similar results (78% and 5% of bases modified in poly(dT) and poly(dC), respectively). Under reaction conditions M in poly(dG), 5% bases were modified whereas no modification of poly(dA) was detected. The extent of modification of poly(dC) and poly(dG) was determined only DPSV, as no changes in the absorption spectra around 310 nm (characteristic of the Os,bipy-

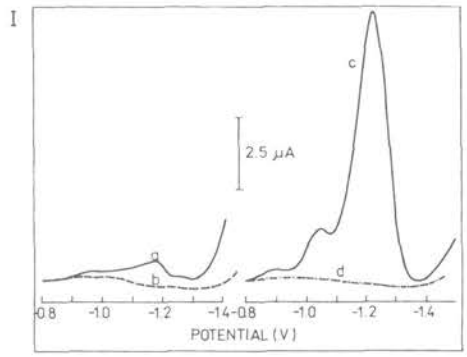

Figure 4. DP stripping voltammograms of (a) poly(dC) and (c) poly(dT) modified with Os,bipy under mild conditions. (b) background electrolyte; (d) unmodified poly(dT). 2×10^{-4} mol/l polynucleotides in 0.05 mol/l sodium phosphate (pH 7.0) were treated with 3×10^{-4} mol/l osmium tetroxide, 3×10^{-4} mol/l bipy for 2 hours at 26 °C, and the reaction products were separated at a Sephadex G-25 column NAP-5. DPSV measurements were performed at a concentration of 0.9 μg/ml with the accumulation time 90 s; for other conditions see legend to Fig. 2.

DNA adducts) were observed (due to the low extent of modification (Fig. 2)).

It is known that at neutral pH and room temperature the content of the ordered (base stacked) structure in single-stranded poly(dC) is much higher than that in poly(dT). To learn whether the observed difference in reactivity of poly(dT) and poly(dC) (Tab. I, Fig. 4) was not due only to the structure differences, we modified these polynucleotides at elevated temperature (4×10^{-4} mol/l polynucleotide, 1×10^{-3} mol/l Os,bipy, 3 min, 90 °C in SSC/20), i.e. under conditions where both polynucleotides are practically structureless. Under these conditions 61% of residues in poly(dT) were modified, whereas modification of poly(dC) was not detectable (not shown).

Nucleotide sequencing: Selective cleavage at thymidine sites due to treatment of single-stranded DNA with Os,py or Os,bipy under mild reaction conditions. We were interested in whether the selectivity of osmium tetroxide for thymines observed under mild reaction conditions (Tab. I) would be manifested as specific cleavage at thymidine sites in the Maxam and Gilbert sequencing method with Os,bipy or Os,py used as reagents. DNA labelled with ^{32}P on one of the 5' ends was thermally denatured and reacted with 1×10^{-3} mol/l OsO_4, 1% pyridine or 1×10^{-3} mol/l Os,bipy 4 or 16 min at 24 °C. The samples were then treated according to the standard Maxam and Gilbert (1980) procedure. After 4 min treatment of DNA with Os,bipy or 16 min treatment with Os,py, specific cleavage at thymidine residues was observed (Fig. 5*A* lanes, *B* lanes). The pattern obtained after 4

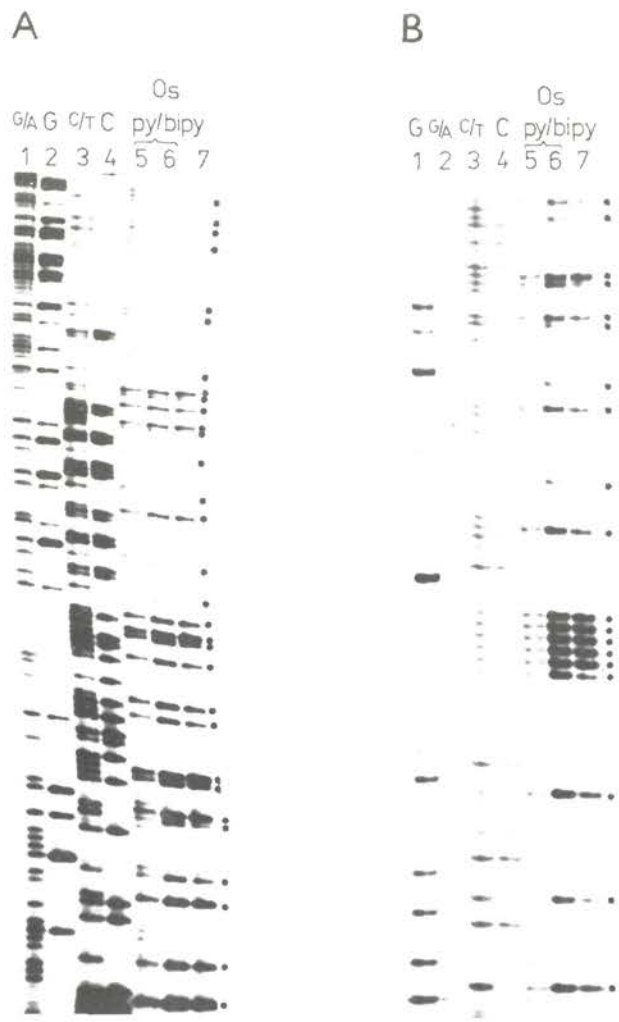

Figure 5. Use of Os,py and Os,bipy in nucleotide sequencing. Denatured DNA was treated with 1×10^{-3} mol/l OsO_4, 1% pyridine (lanes 5,6) or with 1×10^{-3} mol/l OsO_4, 1×10^{-3} mol/l bipy (lane 7) for 4 min (lanes 5,7) or 16 min (lane 6) and separated in 5% (A) or 8% (B) denaturing polyacrylamide gels. For details see Materials and Methods. Positions of T residues are indicated with dots. Good results were also obtained under other modification conditions such as 6×10^{-4} mol/l OsO_4, 1.2% pyridine (or 6×10^{-4} mol/l bipy) for 15 min at 26 °C (Matyášek 1990).

min treatment with Os,py was less specific showing in addition to T's also some cleavages at G residues. Under the reaction conditions resulting in the specific modification of T's in single-stranded DNA practically no effect was observed with double-stranded DNA (not shown).

Piperidine cleaves at the sites of osmium modified T, C and G residues in DNA. We attempted to learn whether modifications of C and G residues in DNA result in the sugar-phosphate backbone cleavage due to piperidine and heat. Poly(dC), poly(dG) and poly(dT) were ^{32}P end-labelled and treated with Os,bipy to obtain roughly the same extent of modification in each polynucleotide (poly(dC) and poly(dG): 1×10^{-3} mol/l Os,bipy, 15 min at 26 °C; poly(dT): 4×10^{-4} mol/l Os,bipy, 20 min at 0 °C). This treatment resulted in modification of about 3% of all polynucleotide bases as determined by DPSV. The modified samples were then treated with 1 mol/l piperidine for 30 min at 90 °C and electrophoresed. As a result of this treatment all three polynucleotides were strongly degraded (not shown). Due to polydispersity of the polynucleotide samples no attempt was made to evaluate the results quantitatively. For poly(dA) same modification conditions as for poly(dG) were used; no degradation of poly(dA) was, however, observed upon this treatment. Poly(dC) and poly(dT) modified with 1×10^{-3} mol/l OsO_4, 2% pyridine (15 min at 0 °C) were degraded by piperidine treatment (not shown), similarly to the Os,bipy modified polynucleotides.

Discussion

Different reactivity of bases towards Os,bipy. In this work we show that Os,bipy at millimolar concentrations and short incubation times (used usually in DNA structure probing) reacts with thymine residues in DNA more than ten times faster than with those of cytosine (Tab. I, Fig. 4). Earlier, a high selectivity for thymines was also observed in DNA oxidation with osmium tetroxide alone (Burton and Riley 1966; Beer et al. 1966) and with potassium permanganate (Jones and Walker 1963; Hayatsu and Ukita 1967). Long incubation with Os,bipy resulted in a strong cytosine modification comparable to that of thymine (Tab. I). Strong reaction conditions and long incubation times (1×10^{-3} mol/l OsO_4, 3 mol/l pyridine or 9×10^{-3} mol/l OsO_4, 9×10^{-3} mol/l bipy with a 1×10^{-3} mol/l polyribonucleotide (or DNA) for 24 or more hours at room temperature) were applied by Chang et al. (1977) who demonstrated modification of all pyrimidine bases in denatured DNA as well as full modification of poly(rC) with Os,bipy and Os,py. These authors observed no reaction of Os,bipy with poly(rG) and poly(rA) under the same conditions.

We have shown for the first time that Os,bipy modifies in addition to thymine and cytosine also guanine residues. The relatively small extent of poly(dG) modification at strong reaction conditions S (Fig. 3, Tab. I) might be connected with the effect of multistrand structure of this polynucleotide on the accessibility of bases for the reaction with Os,bipy. The negative result obtained by Chang et al. (1977) with poly(rG) was most probably due to lower sensitivity of the absorption spectrophotometry as compared to DPSV used in this work. Recently, the ability of osmium tetroxide to react with imidazoles was demonstrated (Kobs and Behrman

1987), the imidazole ring might thus represent the Os,bipy reaction site in guanine. Futher work will, however, be necessary to better understand the guanine reaction with Os,bipy.

Use of osmium tetroxide in nucleotide sequencing. In their original paper, Maxam and Gilbert (1977) described reactions based on the application of dimethyl sulphate and hydrazine that cleaved DNA selectively at G, G>A, A>G, A>C, C+T and C; later, methylene blue based cleavage at T was introduced by Friedmann and Brown (1978). In contrast to dimethyl sulphate, which reacts equally well with both single- and double-stranded DNAs, Os,py under certain conditions reacts rapidly with single-stranded DNA whereas, no reaction occurs in double-stranded B DNA (Paleček et al. 1990). Nevertheless, under the conditions used by Friedmann and Brown (i.e. 3.125% osmium tetroxide with 6.25% pyridine) T residues in double-stranded DNA were modified (Friedmann and Brown 1978). This result suggests that under these conditions the DNA double helix was secondarily opened, which is in agreement with our finding (Lukášová et al. 1982; 1984; Vojtíšková et al. 1985) that high osmium tetroxide,pyridine concentrations induce disruption of the DNA secondary structure. On the other hand, no test has ever been made of how complete this disruption is and whether, e.g., isolated AT pairs in GC-rich blocks do not resist the Os,py denaturing action. The influence of the secondary structure on the accessibility of T residues for the osmium reagent was also considered by Friedmann and Brown (1978). We therefore suggest first to denature DNA gently, and then to modify it in single-stranded form with Os,py under the mild conditions such as those given in Fig. 5. Using Os,bipy instead of Os,py may represent a better alternative, as Os,bipy adducts with nucleic acids are more stable (Chang et al. 1977; Paleček et al. 1987c) and transesterification reactions (which cannot be excluded in Os,py adducts) practically do not occur.

In some experiments in which Os,py reagent was applied to probe DNA by other authors (Johnston and Rich 1985; Johnston 1988b) concentrations of Os,py corresponded to or were only slightly lower than those used by Friedmann and Brown (1978). Such concentrations may induce changes in DNA conformation and should therefore be avoided in DNA structure probing.

Probing of DNA secondary structure with osmium tetroxide. When we used Os,py or Os,bipy as probes of DNA structure (references in Paleček et al. 1990) the reaction conditions were close to the mild conditions M used in this work. Under these conditions bases contained in structures with single-stranded character (e.g. bases in the cruciform loop) were site-specifically modified (e.g. Lilley and Paleček 1984), while the bases contained in the intact B DNA remained unmodified. Detection of osmium modified bases at single-nucleotide resolution showed predominantly modified thymines, and exceptionally also some cytosines, guanines and adenines (Johnston and Rich 1985; McClellan et al. 1986; Galazka et al. 1986; 1987; John-

ston 1988a,b; Htun and Dahlberg 1988; Hanvey et al. 1988b; Nejedly et al. 1988; 1989). Bases available for the reaction in a supercoiled DNA (e.g. bases in the cruciform loop) usually constitute less than 1% of all bases in the molecule. Thus, while the concentration of total DNA was roughly the same as under conditions M the concentration of reactive bases was more than 100 times lower than in the experiments with single-stranded DNA. Such a large excess of the osmium probe might result in modification of C and G (in addition to T) residues if they are available for the reaction. The observed modification of A is difficult to explain at the present time, as the reaction of purine bases with Os,py and Os,bipy is poorly understood.

Abbreviations used: bipy - 2,2' bipyridine; Os,py - OsO_4, pyridine; Os,bipy - OsO_4, 2,2'-bipyridine; DPSV - differential pulse stripping voltammetry; HMDE - hanging mercury drop electrode; SSC/20 - 7.5×10^{-3} mol/l sodium chloride with 7.5×10^{-4} mol/l sodium citrate, pH 7.

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