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Reinterpretation of fluorescence of terbium ion-DNA complexes

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Terbium (Tb^{3+}) fluorescence was used to investigate local non-denaturation perturbations of double-helical DNA structure induced in this nucleic acid by various physical and chemical agents. It has been shown that the interaction of Tb^{3+} with DNA into which single-strand or double-strand breaks have been introduced by DNase I or by low doses of ionizing radiation does not influence the fluorescence of the lanthanide cation. On the other hand, interaction of terbium with DNA modified by the antitumour drug *cis*-diamminedichloroplatinum(II) at low levels of binding and by low doses of ultraviolet radiation (wavelength 254 nm) has been shown to result in substantial enhancement of the fluorescence of this cation. It has been proposed that the terbium fluorescent probe can also be exploited successfully for the purpose of analysing the guanine bases present in distorted double-stranded regions of DNA, in which only the vertical stacking of the base-pairs is altered.

1. Introduction

Fundamental information on the general features of biomacromolecules' conformation has been obtained in particular by means of X-ray crystallographic techniques, NMR spectroscopy and other physical methods. However, the use of these technique has a number of limitations, since such methods usually cannot furnish information about a small region of the biopolymer, the structural properties of which are different from those of the remainder of the macromolecule. Among techniques suitable for the analysis of local conformational changes are those utilizing chemical probes [1–5]. An apparent property of such probes is their ability to react only with a small but altered part of the biopolymer.

A number of chemical probes have been used in research into nucleic acids, which differ in reactivity to the various groups in these biomacromolecules. A considerable body of experimental evidence suggests that Tb^{3+} can be used as a chemical probe of nucleic acid secondary structure [6–13]. This trivalent lanthanide exhibits a low level of intrinsic fluorescence in aqueous solution that is greatly enhanced on chelation to organic ligands, such as the aromatic rings of nucleic acid bases, with which it forms energy-transfer complexes. Guanine induces the strongest enhancement, particularly when this purine is in a polynucleotide form. In contrast, it has so far been generally accepted that base-paired residues do not give rise to fluorescence enhancement.

The application of Tb^{3+} to analysis of nucleic acid structure requires sufficient data on its binding to specific conformations of these biomacromolecules. Moreover, a great advantage of chemical probes, which are usually represented by small molecules, is the low probability of their being able to bring about significant alterations in nucleic acid structure before reacting with a nucleotide residue. These undesired phenomena

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occur, for instance, when single-strand-specific nucleases are used in studies of local conformational alterations in nucleic acids. Nevertheless, the conclusion was published recently [14] that at low ionic strength, Tb^{3+} binding markedly alters the secondary structure of DNA, which could cause serious limitations to the use of this probe in analysing the conformation of DNA.

It is the purpose of this work to reinvestigate the effect of Tb^{3+} on DNA conformation, and also to specify more precisely the spectrum of local conformations with which this lanthanide cation interacts in such a manner that its fluorescence is enhanced.

2. Materials and methods

Polydisperse linear fragments of calf thymus DNA were isolated and characterized as in our previous papers [15,16]. Single-strand breaks were introduced into DNA by DNase I (California Corp. for Biochemical Research): a sample of DNA at 0.15 mg/ml in 0.01 M $MgSO_4$ with 0.01 M NaCl plus 0.02 M sodium phosphate (pH 7.0) was incubated with 1×10^{-8} g DNase I/ml. The enzymatic reaction was stopped by the addition of 1/40 vol. of 1.0 M sodium citrate and cooling the sample in an ice bath. Before analysis, these DNase I-treated samples were exhaustively dialyzed against 20 mM Tris-HCl buffer (pH 7.3). The introduction of single-strand breaks into DNA was monitored by means of differential pulse polarography (DPP) [17,18]. The increase in DPP peak II of DNA under conditions when no change in viscosity occurred was taken as qualitative evidence of the introduction of new single-strand breaks [17,18]. Incubation with DNase I for more than 20 min resulted in a decrease in viscosity, indicating that double-strand breaks also occurred. Our samples of DNA treated with DNase I for a period of about 20 min contained roughly 0.1–0.2% of single-strand breaks, based on the fact that this number of interruptions of phosphodiester bonds by DNase I produces no change in viscosity [19,20].

DNA samples were γ-irradiated in 20 mM Tris-HCl buffer (pH 7.3) at a nucleic acid concentration of 0.30 mg/ml, in the presence of atmospheric oxygen using a ^{60}Co source. A germicidal lamp (Philips TUV 15 W; maximum emission at 254 nm) was used for ultraviolet irradiation of DNA at the same concentration and in the same medium as those used in the case of γ-irradiation. Other details of the irradiation procedures have been published elsewhere [21].

cis-Diamminedichloroplatinum(II) (*cis*-DDP) and its *trans* isomer (*trans*-DDP) were synthesized and characterized at the Institute of Pure Chemicals (Lachema Brno, Czechoslovakia). Interaction of platinum complexes with DNA was performed in a medium of 0.01 M sodium perchlorate. The composition of Pt-DNA complexes was characterized according to the number of platinum atoms fixed per nucleotide residue r_b; r_b values were measured polarographically [22,23].

DPP curves were recorded with a PARC model 384B polarographic analyzer [24]. Briefly, DPP analysis of denaturation or local conformational alterations in DNA is based on the fact that polarographically reducible bases (adenine and cytosine) present in intact DNA do not yield a reduction current at the dropping mercury electrode. On the other hand, the bases present in double-stranded distorted ('premelted') regions or in denatured regions are assumed to be more accessible and thus able to yield a polarographic current; these bases may give rise to the DPP peak II at around −1.36 V (vs. saturated calomel electrode) in the former case and to the more negative (by approx. 0.15 V) peak III in the latter [18,24]. The lower detection limit of denatured DNA in the samples of double-stranded DNA by means of DPP is 0.5% [18,24]. Other details of the polarographic analysis of nucleic acids can be found in a recent paper [18].

Viscosity was measured with a four-gradient Ubbelohde-type viscometer. Absorption spectroscopic measurements were carried out with a Beckman DU-8B apparatus. Circular dichroism (CD) spectra were recorded at 25° C on a Jobin-Yvon Dichrograph mark IV using 1-cm cuvettes.

Terbium fluorescence measurement were performed as follows: terbium was added to 8 μg treated or control DNA/ml at a final concentration equivalent to twice the monomeric nucleotide

content. The fluorescence intensity was measured after equilibration for 60 min at 25 °C in the dark, by which time the binding of terbium is known to be complete [12]. Fluorescence intensity was measured using a 1 cm quartz cell in a Shimadzu RF 40 spectrofluorophotometer equipped with a high-pressure xenon lamp. The excitation wavelength of 290 nm and emission wavelength of 546 nm were optimal for maximizing energy transfer and minimizing background light scattering. The entrance and exit slit widths were maintained at 5 nm. $TbCl_3 \cdot 6H_2O$ (Wako Pure Chemicals) was made up to 10 mM in distilled water as determined by titration with EDTA [25].

3. Results and discussion

The conformation of DNA modified by Tb^{3+} has already been studied using optical methods. It was shown [14] that the CD spectrum of double-helical DNA in 1 or 10 mM cacodylate buffer (pH 6.5) was strongly altered upon addition of terbium. This observation has cast doubt on the suitability of Tb^{3+} for probing DNA secondary structure. Fig. 1 compares the CD spectra of DNA in the presence of Tb^{3+} in the case where the concentration of Tb^{3+} was 1.4-times that of the monomeric

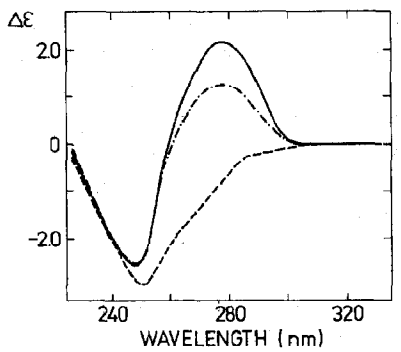

Fig. 1. CD spectra of Tb^{3+}-DNA complexes. DNA concentration was 0.026 mg/ml. (———) DNA in 20 mM Tris-HCl buffer (pH 7.3) or 10 mM cacodylate buffer (pH 6.5); no terbium was added. (·–·–·) DNA in 20 mM Tris-HCl buffer (pH 7.3); Tb^{3+} concentration was 1.4-times the monomeric nucleotide content. (- - - - -) DNA in 10 mM cacodylate buffer (pH 6.5); Tb^{3+} concentration was 1.4-times the monomeric nucleotide content.

nucleotide. When 1–20 mM cacodylate buffer (pH 6.5) was used, the CD spectrum was influenced very strongly by the addition of Tb^{3+} [14]. On the other hand, when 20 mM Tris-HCl buffer (pH 7.5) was used as the background electrolyte, the CD spectrum of DNA exhibited markedly smaller alterations upon addition of Tb^{3+}. The effect of medium on the efficacy of Tb^{3+} in altering DNA conformation has not been investigated in detail. Nevertheless, it is evident from the results so far obtained [6–13] that Tb^{3+} can influence only the overall conformation of DNA, without changing the number of base residues capable of reacting with the lanthanide cation in such a way that this interaction would result in striking enhancement of terbium fluorescence. In order to minimize any effect of Tb^{3+} on the overall conformation of DNA, 20 mM Tris-HCl buffer (pH 7.3) was employed throughout this work.

Until recently it was assumed [6–13] that Tb^{3+} is a fluorescent probe only for the DNA regions that contain unpaired bases, i.e., for denatured regions, in which hydrogen bonds between complementary base residues are ruptured. We therefore attempted to determine whether this fluorescent probe can also reveal bases present in DNA regions that are distorted in a different manner from denaturation.

A simple non-denaturation modification of DNA secondary structure, which results only in local distortion in this biomacromolecule, may be the introduction of single-strand breaks. It has been shown [17,26] that local perturbation of the double-helical DNA conformation occurs near single-strand breaks. Our first endeavour was to ascertain whether single-strand breaks produce a local change of the double-helix, allowing accessibility of terbium to the guanine residue which in turn produces fluorescence enhancement. Single-strand breaks were introduced into DNA by treatment with DNase I for less than 20 min, as described in section 2, and their presence was checked by DPP [17]. When these DNA samples were incubated with terbium no enhancement in fluorescence over the untreated control was observed. On treatment of DNA with DNase I for more than 20 min, the viscosity of the DNA sample started to decrease. This observation indi-

cates that the DNase I treatment of DNA also resulted, under these conditions, in the formation of double-strand breaks (in addition to single-strand breaks). It has been shown [27–29] that non-denaturation conformational alterations may also occur in the region of the ends of the DNA molecule. When DNA treated with DNase I for periods longer than 20 min was incubated with terbium, no change in lanthanide cation fluorescence was observed. The results obtained with DNA samples treated with DNase I support the view that neither single- nor double-strand breaks produce local perturbations of the double-helical DNA structure which would allow terbium to react with guanine residues with the consequent increase in fluorescence of the lanthanide cation.

DNA damaged by low doses of ionizing radiation was also used to demonstrate whether the local non-denaturation distortions induced by this physical agent [21,27,28] result in fluorescence enhancement for Tb^{3+}. The appearance of local non-denaturation distortions in DNA due to γ-irradiation was again checked by DPP [21,27]. It has been shown that single- and double-strand breaks, and chemically altered bases are the most frequent lesions in DNA damaged by low doses of ionizing radiation [27,29]. In this case, as well, no increase in accessibility of terbium to guanine residues of DNA resulting in fluorescence enhancement was observed.

The terbium probe fluoresces primarily with guanine residues [7], and thus detects any perturbations in the regions of DNA that contain particularly a G-C pair. No effect of single- or double-strand breaks in DNA could be detected with the terbium probe. This suggests that the DNA double helix is essentially geometrically continuous at least across nicks or at the ends of the molecule close to G-C pairs. This conclusion is consistent with the observation that irradiation of synthetic polynucleotides, poly(dG-dC) · poly(dG-dC), by ionizing radiation does not result in local distortions detectable by DPP [33].

Modification brought about by the antitumour drug *cis*-DDP is also a factor which induces local non-denaturation distortions in DNA at relatively low levels of binding [24]. It has previously been shown [20,34] that this drug affects DNA in such

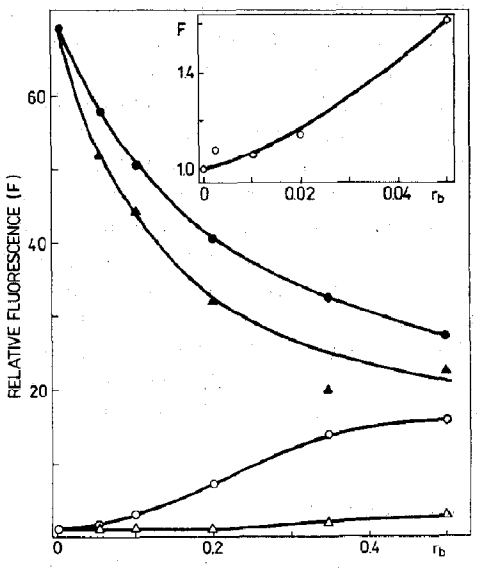

Fig. 2. Change in fluorescence produced by varying concentrations of *cis*-DDP (○, ●) and *trans*-DDP (△, ▲) (expressed by means of r_b) with 8 μg DNA per ml. (○, △) Double-helical DNA; (●, ▲) thermally denatured DNA. Fluorescence of untreated control was arbitrarily set at unity.

a way that the modification also results in a marked enhancement of Tb^{3+} fluorescence. In contrast, the results obtained to date describing modification of DNA by *trans*-DDP, which does not display antitumour activity, with the aid of terbium probe are contradictory (cf. refs. 8 and 34). Whereas Arquilla et al. [8] reported no change in terbium fluorescence upon addition to *trans*-DDP-modified DNA, Houssier et al. [34] later observed a considerable increase in Tb^{3+} fluorescence. We therefore re-investigated Tb^{3+} fluorescence upon addition to platinum-DNA complexes (fig. 2); our results confirmed those of Arquilla et al. [8]. We propose that the difference between the results of Houssier et al. [34] on the one hand and those of Arquilla et al. [8] and ours on the other could be connected with inappropriate handling of the DNA stock solution by the former authors [34].

trans-DDP (in contrast to *cis*-DDP) does not give rise to changes in double-helical DNA on binding, resulting in a greater number of guanine residues capable of interacting with Tb^{3+} in a

manner conductive to the enhancement of lanthanide fluorescence (ref. 8 and fig. 2). This result was rather surprising, because *trans*-DDP (in contrast to *cis*-DDP), as a result of binding to double-helical DNA, induces more severe conformational changes in this nucleic acid, with behaviour characteristic of denaturation even at low levels of binding ($r_b = 0.02$) [24]; such a conformational alteration was expected to cause a considerable increase in Tb^{3+} fluorescence. The platination of thermally denatured DNA by both *cis*-DDP and *trans*-DDP resulted in marked inhibition of Tb^{3+} fluorescence (fig. 2). The results obtained were not influenced by energy transfer between the platinum complex and Tb^{3+} in solution based on the diagnostic tests and criteria described in recent reports [8,35]. Therefore, inhibition of Tb^{3+} fluorescence due to binding of both platinum complexes indicates competition between the platinum complexes and Tb^{3+} for the N(7) atom of single-stranded guanine residues; guanine residues in single-stranded DNA react with platinum drugs such that the drug fixed to DNA hinders or displaces Tb^{3+} from the electron donor group of the guanine residue. Our results thus support the view that the negligible enhancement of Tb^{3+} fluorescence in the case of *trans*-DDP-modified double-helical DNA could be due to at least two phenomena with opposite effects on fluorescence enhancement of the lanthanide ion: on the one hand, denaturation, resulting in an increased proportion of guanine residues accessible to reaction with terbium ion in a manner conducive to enhancement of the lanthanide ion's fluorescence, and on the other, displacement of Tb^{3+} from its second site on the guanine residues by bound drug resulting in inhibition of Tb^{3+} fluorescence, as in the case of thermally denatured DNA. In other words, the binding of *trans*-DDP to double-helical DNA probably results in the formation of denatured segments of DNA in which an overwhelming majority if not all single-stranded guanine residues are bound to *trans*-DDP, so that terbium is no longer able to react with this base to produce fluorescence enhancement. Displacement of Tb^{3+} from single-stranded guanine residues was also observed in the case of thermally denatured DNA modified by *cis*-DDP (fig. 2). In this respect, however, *cis*-DDP was significantly less effective than the *trans* isomer. The observation that modification of double-helical DNA by *cis*-DDP results in marked enhancement of Tb^{3+} fluorescence (fig. 2) now seems to be consistent with the hypothesis that the fluorescence enhancement of Tb^{3+} produced by conformational alterations induced in the DNA by *cis*-DDP dominates over the decreased fluorescence intensity caused by the displacement of terbium from guanine residues by *cis*-DDP.

The results of DPP and CD analyses of *cis*-DDP binding to double-helical DNA have revealed [24] that at low levels of binding ($r_b \leqslant 0.02$) this drug induces local, less severe non-denaturation alterations in the conformation of this biomacromolecule. The main candidate for this conformational change is an intra-strand cross-link between two adjacent guanine or guanine and adenine residues. NMR studies have demonstrated [39,40] that the appearance of these intra-strand cross-links does not result in the rupture of hydrogen bonds, and that only significant changes in vertical stacking of base-pairs around the site of platination occur. As shown in the inset to fig. 2, this kind of non-denaturation conformational change induces a small but significant increase in Tb^{3+} fluorescence. Therefore, it appears reasonable to conclude that not only unpaired single-stranded bases but also bases present in double-helical segments in which vertical stacking of the base-pairs is significantly changed (without rupture of hydrogen bonds) can be detected by terbium fluorescence.

Local changes in the secondary structure of DNA, which differ markedly from those evoked by denaturation, also occur after low doses of ultraviolet radiation of wavelength 254 nm ($\sim 1 \times 10^3$ J/m^2) [21,31]; conformational changes of a different nature including denaturation take place in DNA only after relatively higher doses of ultraviolet radiation (above approx. 1×10^4 J/m^2) [21,31].

The presence of double-stranded, distorted but non-denatured regions in our DNA samples ultraviolet-irradiated at doses of $0.5–7.0 \times 10^3$ J/m^2 with the simultaneous absence of denatured regions was demonstrated by DPP [21,31]. When

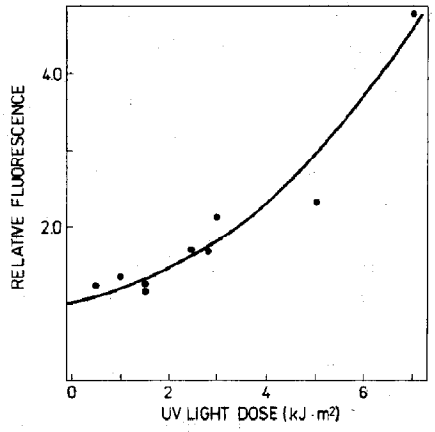

Fig. 3. Fluorescence enhancement induced by varying doses of ultraviolet radiation with 8 μg double-helical DNA per ml. Fluorescence of non-irradiated control was arbitrarily set at unity.

such ultraviolet-irradiated samples were incubated with terbium, enhancement of its fluorescence over that of the non-irradiated control was observed (fig. 3).

A wide variety of photoproducts are formed in ultraviolet-irradiated DNA. In double-helical DNA, the major products are pyrimidine dimers (more than 70% of the total number of photoproducts) [38]. It is reasonable to expect that the formation of pyrimidine dimers results primarily in marked changes in the vertical stacking of base-pairs around the dimer, similar to those observed around the intra-strand cross-link induced by bifunctional binding of *cis*-DDP.

4. Conclusions

Tb^{3+} is a sensitive fluorescent probe of guanine bases present not only in single-stranded denatured segments of DNA but also in local, non-denatured distortions of double-helical DNA, in which hydrogen bonds can be preserved but vertical stacking of base-pairs is altered. Moreover, the terbium fluorescent probe is not suitable for detection of single-strand or double-strand breaks in DNA, which in turn implies that local perturbations of the double-helical structure near nicks or ends of DNA molecules do not involve unpaired guanine bases or marked changes in the vertical stacking of G-C pairs.

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