Structural specificity effects of trivalent polyamine analogues on the stabilization and conformational plasticity of triplex DNA

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Natural polyamines, i.e. putrescine, spermidine and spermine, are excellent promoters of triplex DNA. Using melting temperature (T_m) measurements and CD spectroscopy, we found that structural alterations on spermidine backbone, including methylation, or acetylation at the N¹-, N⁴- and/or N⁸-positions had a profound influence on the stability and conformation of poly(dA).2poly(dT) triplex. The conformation of the polynucleotide complex underwent sequential changes from B-DNA to triplex DNA as the concentration of spermidine increased from 0 to 50 μ M in a buffer containing 10 mM sodium cacodylate and 1 mM EDTA (pH 7.2). At 60 μ M spermidine, the CD spectrum of triplex DNA was comparable with that of Ψ -DNA, with a strong positive band centred around 260 nm. A negative band was also found at 295 nm. At higher concentrations of spermidine, however, the intensity of the positive band progressively decreased and the peak intensity was found at a 1:0.3 molar ratio of DNA phosphate:spermidine. Temperature-dependent CD

analysis showed that the Ψ -DNA structure melted to single-stranded DNA at temperatures above the T_m determined from the absorbance versus temperature profile. Comparable effects were exerted on the conformation of triplex DNA by $\text{Co}(\text{NH}_3)_6^{3+}$, an inorganic trivalent cation. Substitution of the N⁴-hydrogen of spermidine by a cyclohexyl ring or the fusion of the N⁴-nitrogen in a cyclic ring system, as in piperidine, enhanced the ability of spermidine analogues to stabilize triplex and Ψ -DNA forms over a wider concentration range compared with spermidine. These data demonstrate a differential effect of trivalent cations in stabilizing triplex DNA and provoking unusual conformations such as Ψ -DNA. Synthetic homologues of spermidine that stabilize triplex DNA over a wider range of concentrations than that stabilized by spermidine itself might have potential therapeutic applications in the development of an anti-gene strategy against several diseases, including cancer and AIDS.

INTRODUCTION

Triplex DNA has acquired considerable interest in recent years as an anti-gene strategy to suppress the transcription of disease-related genes [1–5]. Promoter or enhancer regions of a large number of genes, including *c-myc*, *erb-2*, interleukin-2 receptor, dihydrofolate reductase, etc., have DNA sequences capable of forming triplex DNA [6,7]. A major challenge in developing the triplex-DNA-based anti-gene strategy lies in the design of appropriate ligands to stabilize triplex DNA under physiological ionic and pH conditions. Using poly(dA).2poly(dT) synthetic triplex DNA as a model system, we found a remarkable selectivity of natural polyamines and their synthetic homologues in stabilizing triplex DNA [8]. Polyamine-mediated triplex DNA stabilization has been reported by other investigators as well [9,10].

Despite intense efforts to identify ligands and other conditions necessary for the stabilization of triplex DNA, currently available information on the conformation and/or conformational dynamics of this form of DNA structure is scant [11–14]. A characteristic negative CD band around 210 nm has been commonly observed in the CD spectrum of triplex DNA [15]. In poly(dA).2poly(dT) triplex, an increase in the intensity of the positive band at 250 to 300 nm and the induction of a negative band at 300 to 350 nm were also found in the presence of intercalating drugs such as ethidium bromide [16]. Polyamines are known to provoke several structural and conformational transitions in duplex B-DNA, including condensation and aggregation, as well as the induction and stabilization of A-DNA,

Z-DNA and Ψ -DNA conformations [17–21]. Since duplex DNA is capable of undergoing these structural transitions, we questioned whether triplex DNA could assume similar structural/conformational variants. Furthermore, a recent X-ray crystallographic analysis of a triplex DNA formed by a peptide nucleic acid–DNA complex revealed the existence of a previously unknown P-form helix and suggested the potential existence of additional classes of stable DNA triplexes yet to be discovered [22]. For the present study, we focused on spermidine, the trivalent polyamine and a series of its structural analogues, including acetylated spermidines, alkylated spermidines and cyclic spermidines. These compounds exert different degrees of ionic and steric effects on DNA recognition and thus provide an excellent model system to delineate the structural effects of polyamines on DNA structure and conformation, and to elucidate the mechanism of polyamine–DNA interactions.

Previous studies have shown a structural specificity effect of spermidine and its homologues of the general structure, $\text{H}_2\text{N}(\text{CH}_2)_n\text{NH}(\text{CH}_2)_3\text{NH}_2$ (where $n = 2–8$; $n = 4$ for spermidine), on the induction and stabilization of Z-DNA and triplex DNA, as well as on protein–DNA interactions [8,19,23]. This structural specificity of polyamines has important implications in developing a triplex-DNA-based anti-gene strategy, as found in our recent studies on the transcriptional suppression of *c-myc* oncogene by a triplex-forming oligonucleotide in an oestrogen receptor-positive breast cancer cell line, MCF-7 [5]. In the present study, we synthesized several spermidine analogues that maintained the positive charge spacing unaltered, whereas steric

Abbreviations used: T_m , melting temperature; 1,3-APAMPP, 1-[(3-aminopropyl)-4-aminomethyl]piperidine.3HCl; 4-N-3-APAMPP, 4-[(N-3-aminopropyl)aminomethyl]piperidine.3HCl; TFO, triplex-forming oligonucleotide.

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constraints were introduced to differentiate ionic versus structural effects in the binding of polyamines with DNA. The effects of these compounds on the helix-coil transitions of duplex and triplex forms of poly(dA).poly(dT) and poly(dA).2poly(dT), respectively, were determined using melting temperature (T_m) measurements. In addition, we monitored the conformational transitions exerted by these compounds on poly(dA).2poly(dT) triplex using CD spectroscopy. A remarkable structural specificity effect of spermidine analogues was observed in their ability to stabilize triplex and to provoke Ψ -DNA. Alkylation and cyclization of the pendant amino groups increased the concentration range over which triplex and Ψ -DNA structures were stabilized; however, acetylation markedly reduced the ability of spermidine to provoke and stabilize the Ψ -DNA structure.

EXPERIMENTAL

Polyamines

Spermidine.3HCl, N^1 -acetylspermidine.2HCl, and N^8 -acetylspermidine.2HCl were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). $\text{Co}(\text{NH}_3)_6\text{Cl}_3$ (> 99.99%) was purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.) and used without further purification. Homospermidine.3HCl was prepared by methods described previously [8]. N^1, N^8 -Diacetylspermidine.HCl, N^1 -methylspermidine.3HCl, N^8 -methylspermidine.3HCl, N^4 -methylspermidine.3HCl, N^1, N^8 -bis(ethyl)spermidine.3HCl, N^1 -cyclohexylspermidine.3HCl, 1-[(3-aminopropyl)-4-aminomethyl]piperidine.3HCl (1,3-APAMPP) and 4-[(N -3-aminopropyl)aminomethyl]piperidine.3HCl (4- N -3-APAMPP) were synthesized by us [24–26]. Others were synthesized according to the method for spermidine using N -methyl-

N -(3-bromopropyl)toluene sulphamide (for N^1 -methylspermidine) or cyclohexylamine instead of benzylamine (for N^1 -cyclohexylspermidine). N^1, N^8 -Diacetylspermidine.HCl was synthesized by acetylation of spermidine with acetic anhydride and purified by ion-exchange chromatography with Amberlite CG-50. The structures and purities of all spermidine analogues were confirmed by elemental analysis, NMR, HPLC and GC-MS. Figure 1 shows the chemical structures of spermidine and its analogues with alkyl substitution and cyclization. Concentrated solutions of the polyamines were prepared in 10 mM sodium cacodylate buffer, pH was adjusted to 7.2, and small volumes were added to polynucleotide solutions to make up the necessary concentrations.

Polynucleotides

Poly(dA) and poly(dT) were purchased from Pharmacia Biotechnology (Piscataway, NJ, U.S.A.). The polynucleotides were dissolved in 10 mM sodium cacodylate buffer (pH 7.2) and 0.5 mM EDTA, and dialysed extensively from the same buffer. All experiments were conducted in this buffer. Concentrations of poly(dA) and poly(dT) were measured using molar nucleotide absorbance coefficients of 8900 at 257 nm for poly(dA) and 9000 at 265 nm for poly(dT). To prepare triplex solution, poly(dA) and poly(dT) were mixed in 1:2 molar ratio in 10 mM sodium cacodylate buffer and the appropriate concentrations of polyamines were added. The concentration of polynucleotide solution used in UV and CD measurements was in the range of $(1.5\text{--}2.5) \times 10^{-5}$ M DNA phosphate. The solutions were heated at 90 °C for 5 min, cooled to room temperature (22 °C), and then allowed to equilibrate for 16 h at this temperature before use in T_m experiments.

T_m measurements

Absorbance versus temperature heating curves were obtained using a Perkin Elmer Lambda 2 spectrophotometer. The temperature of the five-cell holder was controlled by a thermoelectrically regulated programmer, interfaced to an IBM PS2 computer. Melting profiles were obtained by increasing the temperature at a rate of 0.5 °C per min with the absorbance and temperature being recorded every 30 s. T_m was taken as the temperature corresponding to half dissociation of the complexes and the reproducibility was within ± 0.5 °C. The first derivative, dA/dT (where A is the absorbance and T is the temperature), of the melting curve was computer-generated and was also used for determining the T_m of triplex (T_{m1}) and duplex (T_{m2}) DNA. T_m values obtained from both methods did not differ by > 0.5 °C.

CD spectral measurements

CD measurements were performed on an AVIV model 62 DS spectrophotometer (AVIV Associates, Lakewood, NJ, U.S.A.). Rectangular quartz cuvettes (Uvonic, New York, NY, U.S.A.) with 1-cm optical pathlengths were used. Polynucleotide solutions for CD measurements were prepared in a manner similar to that used for UV melting. The CD spectra were recorded from 350 to 200 nm at 25 °C, and normalized by subtraction of the background scan with buffer. The molar ellipticity was calculated from the equation, $[\theta] = \theta/cl$, where θ is the relative intensity, c is the molar concentration of polynucleotides, and l is the pathlength of the cell in cm.

Temperature-dependent changes in the CD spectra of poly(dA).2poly(dT) in the presence and absence of spermidine were measured using a 5-cell cuvette holder. Temperature of the

Figure 1 Chemical structures of spermidine and its analogues used in this study

cuvette holder was controlled with an AVIV thermoregulated five-compartment cell holder and temperature was controlled to $\pm 0.1^\circ\text{C}$. Each temperature measurement was equilibrated for 5 min. CD spectra were collected at 1.0 nm intervals, with 2-s collection time, at every 10°C from 0 to 90°C .

RESULTS

Effect of spermidine on the T_m and CD spectrum of poly(dA).2poly(dT)

Figure 2(A) shows the absorbance versus temperature profile of poly(dA).2poly(dT) in the presence of 0, 10, 25, 50, 60 and $100\ \mu\text{M}$ of spermidine. In the absence of spermidine, there was only one melting transition (55.4°C) corresponding to the melting of duplex DNA. Two melting transitions were observed at 10, 25, 50 and $60\ \mu\text{M}$ spermidine; the first one corresponding to the melting of the triplex to duplex (T_{m1}) and the second one

Figure 2 (A) UV absorbance versus temperature profiles, and (B) the effect of spermidine concentration on the melting temperatures of triplex (T_{m1} , $\square$) and duplex (T_{m2} , $\blacklozenge$) forms of poly(dA).2poly(dT)

All T_m experiments were conducted in a buffer containing 10 mM sodium cacodylate (pH 7.2) and 0.5 mM EDTA. In (A), spermidine concentrations added to the poly(dA).2poly(dT) solution were as follows: 0 (.....); 10 (thick continuous line); 25 (thin continuous line); 50 (line a); 60 (line b); and 100 (line c) μM .

corresponding to the melting of the duplex (T_{m2}). At $100\ \mu\text{M}$ spermidine, T_{m1} and T_{m2} merged into one T_m , indicating the possible melting of the triplex into single strands. All T_m values were increased by spermidine in a dose-dependent manner, although increase in T_{m1} was more pronounced than that of T_{m2} (Figure 2B).

Figure 3(A) shows the CD spectra of the polynucleotides in the presence of 0, 50, 60, 70, 90 and $100\ \mu\text{M}$ spermidine. CD spectral changes at low (5, 10 and $25\ \mu\text{M}$) spermidine concentrations are included as an inset with expanded y-axis to show the characteristic CD spectral features of triplex DNA. In the absence of spermidine, the CD spectrum of poly(dA).2poly(dT) had a negative band at 250 nm and three positive bands at 220, 260 and 280 nm. These spectral features are similar to that previously reported for the duplex form of this polynucleotide [15,16,27]. In the presence of low concentrations of spermidine, triplex formation was indicated by an inversion of the positive peak centred at 220 nm with a new negative peak at 210 nm (inset). In addition, there was an increase in the positive peak at 260 nm and suppression of the positive peak at 280 nm.

A dramatic change in the CD spectrum of the poly(dA).2poly(dT) triplex was observed at $60\ \mu\text{M}$ spermidine. The two small positive bands at 260 and 280 nm merged into one strong positive band centred around 260 nm. This increase is comparable with that found in Ψ -DNA, an ordered, twisted, tight packing arrangement of the DNA helix [21,28]. In addition, there was a negative peak at 295 nm in the presence of $60\ \mu\text{M}$ spermidine. The negative peak at 210 nm, a characteristic feature of triplex DNA, was converted into a strong positive band at 210–220 nm. The negative band at 250 nm did not undergo significant changes up to $50\ \mu\text{M}$ spermidine, but it merged with the positive band at 260 nm during Ψ -DNA formation in the presence of $60\ \mu\text{M}$ spermidine. A broad negative peak was also found centred around 295 nm. Although this negative peak might have originated from differential scattering of circularly polarized light by DNA aggregates [29], absence of this negative band in Ψ -DNA induced by bis(ethyl)spermidine and other alkylated derivatives

Figure 3 Representative CD spectra of the effect of spermidine on poly(dA).2poly(dT)

(A) CD spectra of poly(dA).2poly(dT) in the presence of 0 ($\circ$), 50 ($\bullet$), 60 (∇), 70 ($\blacksquare$), 90 ($\blacktriangledown$), and $100\ \mu\text{M}$ ($\square$) spermidine were recorded at 22°C in the same buffer as used for T_m measurements. The inset shows the effects of 0 ($\circ$), 5 ($\bullet$), 10 ($\blacktriangledown$), and $25\ \mu\text{M}$ ($\diamond$) spermidine. Only selected spectra are shown for clarity. (B) Effect of spermidine concentration on molar ellipticity at 218 ($\bullet$), 250 ($\square$), and 260 ($\blacklozenge$) nm. Molar ellipticity values plotted are the mean of three separate measurements, with S.D. of $< 15\%$.

(see below) suggests that Ψ -DNA formation is not always associated with aggregation of triplex DNA. Despite these marked changes in the CD spectrum of poly(dA).2poly(dT), it retained the triplex form as judged by the absorbance-temperature profile with two melting transitions (Figure 2A). These data demonstrate that the triplex form of poly(dA).2poly(dT) is conformationally ultrapolymorphic and assumes a variety of conformations in the presence of spermidine. There was no major conformational alteration in the CD spectrum of poly(dA).poly(dT) duplex in the presence of spermidine under the same buffer conditions as used in the present study [27].

Addition of $> 60 \mu\text{M}$ spermidine to poly(dA).2poly(dT) produced a decrease in the intensity of the positive peak centred around 260 nm, with a concomitant reduction of the negative peak at 295 nm. The positive CD peak at 210 nm also underwent a reduction in intensity. A plot of the molar ellipticity at 218, 250 and 260 nm against [spermidine] showed a sharp peak at $60 \mu\text{M}$ spermidine, at a 1:0.3 molar ratio of DNA phosphate:spermidine (Figure 3B). Parallel experiments on UV absorption of spermidine-complexed poly(dA).2poly(dT) showed no reduction in light absorption due to precipitation of the polynucleotides.

Effect of spermidine on the CD spectra of triplex DNA during thermal melting

The complexity of the CD spectrum of poly(dA).2poly(dT) in the presence of spermidine prompted us to analyse it as a function of temperature at every 10°C from 0 to 90°C , after allowing thermal equilibrium at each measurement point. Figure 4 shows representative temperature-dependent CD spectra of poly(dA).2poly(dT) in the absence (Figure 4A) and presence (Figure 4B) of $60 \mu\text{M}$ spermidine. In the absence of spermidine, there was a significant reduction in the intensity of the negative band at 250 nm at 60°C . The shoulder at 260 nm disappeared and minor changes in the shape and the intensity of the 280 nm band occurred at 60°C . The CD spectra of the polynucleotides above this temperature were comparable with that of single-stranded polynucleotides [15]. UV melting analysis showed a T_m value of 55°C for the duplex form of poly(dA).poly(dT). The triplex form of poly(dA).2poly(dT) was not formed in the absence of spermidine.

Figure 4 Temperature-dependent CD spectra of poly(dA).2poly(dT) in the absence and presence of spermidine

CD spectra were recorded at 10°C intervals from 0 to 90°C . Spectra shown are at selected temperatures for clarity. (A) CD spectra of poly(dA).2poly(dT) in the absence of spermidine at 0 ($\circ$), 30 ($\bullet$), 60 (∇) and 90 ($\blacktriangledown$) $^\circ\text{C}$. (B) CD spectra of poly(dA).2poly(dT) in the presence of $60 \mu\text{M}$ spermidine at 0 ($\circ$), 30 ($\bullet$), 60 (∇), 70 ($\blacksquare$) and 90 ($\blacktriangledown$) $^\circ\text{C}$.

The CD spectrum of poly(dA).2poly(dT) complexed with $60 \mu\text{M}$ spermidine showed a transition in the peak intensity of the negative band of 295 nm at 70°C (Figure 4B). A more marked change to a CD spectrum corresponding to that of the single-stranded polynucleotide occurred between 70 and 80°C . In UV melting, T_{m1} and T_{m2} of this polynucleotide-spermidine complex were 70 and 73°C , respectively.

We also recorded the CD spectra of poly(dA).2poly(dT) in the absence and presence of spermidine following the CD melting experiment. The Ψ -DNA spectrum was not reformed during the 3 h time period necessary to equilibrate and record the spectra from 90°C to 0°C . However, the characteristics of the triplex DNA spectrum was reformed within this time period in samples containing $60 \mu\text{M}$ spermidine (results not shown). This would suggest that the Ψ -DNA formation is a kinetically controlled process.

Effect of $\text{Co}(\text{NH}_3)_6^{3+}$ and homospermidine on the stability of triplex DNA

We next examined the effects of an inorganic trivalent cation, $\text{Co}(\text{NH}_3)_6^{3+}$, on the T_m and CD spectra of poly(dA).2poly(dT). Figure 5 shows the T_m values and CD spectra of poly(dA).2poly(dT) in the presence of different concentrations of $\text{Co}(\text{NH}_3)_6^{3+}$. This cation stabilized triplex DNA in the concentration range of 10 – $25 \mu\text{M}$ (Figure 5A). At concentrations $> 25 \mu\text{M}$, there was only one melting transition. Changes in the CD spectra were similar to that found in the case of spermidine, with the Ψ -DNA form stabilized at $40 \mu\text{M}$ $\text{Co}(\text{NH}_3)_6^{3+}$ (Figure 5B). The intensity of the bands at 220 , 260 and 295 nm were generally comparable with those of the Ψ -DNA form stabilized by spermidine. Wavelength-specific molar ellipticity versus $[\text{Co}(\text{NH}_3)_6^{3+}]$ plots showed that the maximal stabilization of the Ψ -DNA occurred at a molar ratio of $1:0.25$ of DNA phosphate: $\text{Co}(\text{NH}_3)_6^{3+}$. This result is consistent with several previous studies showing that $\text{Co}(\text{NH}_3)_6^{3+}$ is capable of exerting a spermidine-like effect on the physicochemical properties of DNA, at concentrations lower than that of spermidine itself [30,31]. In the presence of $100 \mu\text{M}$ $\text{Co}(\text{NH}_3)_6^{3+}$, however, the positive and negative bands of the CD spectrum disappeared, probably due to the condensation of the polynucleotides in the presence of high concentrations of $\text{Co}(\text{NH}_3)_6^{3+}$.

Figure 5 Effect of $\text{Co}(\text{NH}_3)_6^{3+}$ on T_{m1} ($\square$) and T_{m2} ($\blacklozenge$) (A), and on the CD spectra (B) of poly(dA).2poly(dT)

T_{m1} and T_{m2} were determined from absorbance versus temperature curves generated with a UV/VIS spectrophotometer. In (B), $\text{Co}(\text{NH}_3)_6^{3+}$ concentrations are as follows: 0 ($\circ$), 25 ($\bullet$), 40 (∇), 60 ($\blacktriangledown$), and 100 ($\square$) μM .

Figure 6 Effect of homospermidine on T_{m1} ($\square$) and T_{m2} ($\blacklozenge$) (A), and on the CD spectra (B) of poly(dA).2poly(dT)

In (B), homospermidine concentrations are as follows: 0 ($\circ$), 50 ($\bullet$), 100 (∇), 200 ($\blacktriangledown$), and 250 ($\square$) μM .

Figure 7 Effect of 1,8-bis(ethyl)spermidine on T_{m1} ($\square$) and T_{m2} ($\blacklozenge$) (A), and on the CD spectra (B) of poly(dA).2poly(dT)

In (B), 1,8-bis(ethyl)spermidine concentrations are as follows: 0 ($\circ$), 100 ($\bullet$), 250 (∇), 500 ($\blacktriangledown$), 1000 ($\square$), and 2000 ($\blacksquare$) μM .

We also examined the effect of a lower homologue of spermidine, homospermidine [$\text{H}_2\text{N}(\text{CH}_2)_3\text{NH}(\text{CH}_2)_3\text{NH}_2$] on the T_m and CD spectrum of poly(dA).2poly(dT). This compound stabilized triplex DNA at 50 μM concentration (Figure 6A). The CD spectrum of the triplex form changed to that of the Ψ -DNA form at about 100 μM (Figure 6B). The maximal stabilization of the Ψ -DNA occurred at a nucleotide:homospermidine molar ratio of 1:1.2. Thus the efficacy of the ligand in inducing Ψ -DNA conformation was compromised by the shortening of one methylene group from spermidine.

Effect of alkyl and acetyl group substitution of spermidine on triplex DNA

In order to further elucidate the role of structural variations of spermidine on its interactions with poly(dA).2poly(dT), we examined the effect of 1,8-bis(ethyl)spermidine in inducing the different conformations of triplex DNA. This compound has been extensively studied as a potential anti-tumour agent [32]. Bis(ethyl)spermidine stabilized triplex DNA at 10–80 μM in UV melting experiments, and produced a single transition at > 80 μM concentration (Figure 7A). The Ψ -DNA conformation was provoked at concentrations > 250 μM spermidine (Figure 7B). Peak intensity of the Ψ -DNA form occurred at 1000 μM ,

Table 1 Comparative efficacy of spermidine and its analogues in triplex and Ψ -DNA stabilization

Compound	Ionic charge	Duplex/triplex range (μM)*	Ψ -DNA range (μM)†	Concn. at maximal ellipticity (μM)	Molar ratio at maximal intensity‡
$\text{Co}(\text{NH}_3)_6^{3+}$	3+	10–25	40–100	40	0.25
Spermidine	3+	10–60	60–100	60	0.3
Homospermidine	3+	50–70	100–250	200	1.2
N^4 -Methylspermidine	3+	10–50	80–2000	250	1.5
N^1 -Methylspermidine	3+	10–40	90–2000	500	3.0
4- N -3-APAMPP	3+	10–60	60–2000	500	3.0
N^8 -Methylspermidine	3+	10–50	100–2000	750	4.5
1,3-APAMPP	3+	10–100	100–2000	750	4.5
N^4 -Cyclohexylspermidine	3+	10–2000	250–2000	1000	6.0
N^1, N^8 -bis(ethyl)spermidine	3+	10–80	250–2000	1000	6.0
N^1 -Acetylspermidine	2+	250–2000	—	—	—
N^8 -Acetylspermidine	2+	250–2000	—	—	—

* Concentration range representing two-stage transitions, allowing the determination of T_{m1}

and T_{m2} .

† Concentration range representing increased molar ellipticity at 260 to 300 nm region.

‡ Represents molar ratio of DNA phosphate:cation at maximal ellipticity at 260 nm.

corresponding to a molar ratio of 1 DNA phosphate:5.5 bis(ethyl)spermidine. The amplitude of the positive band at 260–300 nm was also 7-fold higher than that induced by spermidine. Furthermore, the negative band at 295 nm was not induced by bis(ethyl)spermidine. This result indicates a significant effect of pendant alkyl group substitution on the ability of spermidine to provoke triplex and Ψ -DNA conformations. Unlike spermidine and homospermidine, bis(ethyl)spermidine maintained high peak intensity of Ψ -DNA spectrum without undergoing condensation or transition to another form of Ψ -DNA, over the concentration range studied (250–2000 μM).

We also investigated a series of spermidine derivatives where methyl groups were introduced at N^1 -, N^4 - or N^8 - positions. Results on the effects of these compounds on T_m values and CD spectra of poly(dA).2poly(dT) are summarized in Table 1. In the presence of N^4 -methylspermidine, triplex DNA stabilization occurred in the concentration range 10–50 μM . In CD spectral measurements, the Ψ -DNA conformation was induced at 100 μM concentration with maximal intensity occurring at 250 μM N^4 -methylspermidine. The amplitude of the 260 nm positive band was 3-fold higher than that provoked at optimal concentrations of spermidine. The negative band at 295 nm observed in the presence of spermidine was absent in the spectrum induced by N^4 -methylspermidine. Similar results were observed with N^1 - and N^8 -methylspermidine. The concentration range at which triplex DNA to Ψ -DNA-like conformational transition occurred did not vary significantly between these methylated spermidine analogues, although there were differences in the concentration at which maximal molar ellipticity was observed (Table 1). These results show that variant forms of Ψ -DNA are induced by polyamine analogues, depending on their geometry and steric constraints.

Figure 8 Effect of *N*⁴-cyclohexylspermidine on *T*_{m1} (□) and *T*_{m2} (◆) (A), and on the CD spectra (B) of poly(dA).2poly(dT)

In (B), *N*⁴-cyclohexylspermidine concentrations are as follows: 0 (○), 25 (●), 100 (▽), 500 (▼), 1000 (□), and 2000 (■) μM.

In another attempt to relate the Ψ-DNA formation to physiological effects of polyamines, we examined the ability of acetyl-spermidines to induce unusual DNA structures. Acetylation of polyamines is part of a metabolic pathway in the interconversion and efflux of polyamines from the cell [33]. Acetylation is also associated with the elimination of a positive charge. We therefore examined the effect of *N*¹- and *N*⁸-acetylspermidine on the induction and stabilization of triplex and Ψ-DNA conformations using *T*_m measurements and CD spectroscopy. Table 1 shows a comparison of the concentration range at which different spermidine derivatives and homologues stabilized triplex and Ψ-DNA forms. *N*¹- and *N*⁸-acetylspermidine stabilized triplex DNA at 250–2000 μM concentration. Ψ-DNA was not induced at these concentrations. *N*¹,*N*⁸-Diacetylspermidine was incapable of inducing the triplex or Ψ-DNA structures of poly(dA).2poly(dT) (results not shown). In general, alkylation or acetylation of spermidine decreased its ability to induce and/or stabilize the triplex and Ψ-DNA structures.

Effect of a group of novel spermidine analogues on triplex DNA stabilization

Our results on the effects of alkylation and acetylation of -NH₂ and -NH- groups of spermidine on its efficacy to provoke triplex and Ψ-DNA structures suggested the importance of steric factors and ionic charge of the ligand in binding with poly(dA).2poly(dT). In order to further elucidate the structure–activity correlations of spermidine analogues on the induction and stabilization of triplex DNA, we next investigated the effects of a series of compounds with alterations on *N*¹-, *N*⁴- or *N*⁸-nitrogen atoms on the absorbance–temperature profile and CD spectrum of poly(dA).2poly(dT). Figure 8 shows the effects of *N*⁴-cyclohexylspermidine on the *T*_m and CD spectra of poly(dA).2poly(dT). This compound was unique in showing two melting transitions, corresponding to *T*_{m1} and *T*_{m2}, over the concentration range of 10–2000 μM. CD spectra showed characteristics of the triplex form of the polynucleotide up to 100 μM. CD spectrum changed to that of Ψ-DNA conformation at 500 μM. In this case, the optimal positive CD intensity occurred at 275 nm in the presence of 100 μM *N*⁴-cyclohexylspermidine. This compound did not induce a negative band at 295 nm in the Ψ-DNA form.

In the next set of experiments, we examined the effect of incorporating the *N*⁴-nitrogen as a part of a cyclic ring system, as in the piperidine derivative, on the stability and conformation of

Figure 9 Effect of 1,3-APAMPP on *T*_{m1} (□) and *T*_{m2} (◆) (A), and on the CD spectra (B) of poly(dA).2poly(dT)

In (B), 1,3-APAMPP concentrations are as follows: 0 (○), 25 (●), 100 (▽), 250 (▼), 500 (□), 1000 (■) and 2000 (△) μM.

triplex DNA. Figure 9(A) shows the effect of 1,3-APAMPP on *T*_{m1} and *T*_{m2} of poly(dA).2poly(dT). Distinct melting transitions corresponding to the melting of triplex and duplex DNA forms were found up to a 250 μM concentration of this compound. At higher concentrations, only one melting transition was observed. Characteristics of the CD spectrum of triplex DNA were found at a 25 μM concentration of this cation (Figure 9B). At 250 μM and higher concentrations, the CD spectrum assumed the Ψ-DNA conformation. The concentration range stabilizing Ψ-DNA was 250–2000 μM. We found a similar effect with 4-*N*-3-APAMPP on the absorbance–temperature profile and CD spectra of poly(dA).2poly(dT) (Table 1). This compound stabilized the triplex DNA structure at concentrations > 10 μM. The stabilizing range of this compound on triplex DNA form was lower than that of 1,3-APAMPP. CD spectra showed that this compound was capable of inducing the Ψ-DNA-like conformation at a concentration range of 500–1000 μM.

DISCUSSION

Our results demonstrate a remarkable effect of trivalent polyamines in provoking and stabilizing the triplex DNA form of poly(dA).2poly(dT). An important finding of our CD spectroscopic studies is the transition of triplex DNA into a Ψ-DNA structure in the presence of spermidine and its analogues. This transition occurred at concentrations higher than those required to stabilize the triplex DNA form, but the triplex structure was maintained as judged by *T*_m measurements. Using the UV absorbance–temperature profile, we show that the distinct melting transitions for the triplex and duplex forms of poly(dA).2poly(dT) are present only over a narrow concentration range of 10–60 μM for spermidine. At higher concentrations, spermidine induced a CD spectrum with a strong positive band at 260 nm. An inorganic trivalent cation, Co(NH₃)₆³⁺, produced similar effects with higher efficacy than spermidine, provoking the triplex and Ψ-DNA structures at 40 μM concentration. Deletion of one methylene group from the spermidine structure, as in homospermidine, caused a 5-fold increase in the concentration required to provoke the triplex DNA form of poly(dA).2poly(dT), and a 4-fold increase in its concentration required to induce the maximal positive band intensity of the Ψ-DNA form of this polynucleotide. There was a dramatic difference in the concentration range of spermidine (50–60 μM) and that of *N*⁴-methylspermidine (80–2000 μM) in stabilizing the Ψ-DNA structure. Although a broad negative band centred at 295 nm was

Table 2 CD spectral features of poly(dA) and poly(dT)

Spectral parameters for single-stranded, duplex, and triplex forms of poly(dA) and poly(dT) were determined using 100 mM sodium phosphate buffer, pH, 7 [15]. Spectra of duplex poly(dA).poly(dT) measured in 90 mM Tris/HCl buffer (pH, 7.6) containing 20 μ M EDTA [34] had similar features. CD spectra of triplex DNA shown were measured using 1.1 M sodium phosphate buffer, pH 7 [15]. Similar features were reported for measurements conducted in 10 mM phosphate buffer (pH 7) with 0.1 mM EDTA and 0.55 M Na⁺ by Park and Breslauer [35]. Ψ -DNA data are from this study conducted in a buffer containing 10 mM sodium cacodylate (pH 7.2) and 0.5 mM EDTA.

Species	Major CD bands:		
	Wavelength (nm)	Sign of ellipticity	10 ⁻⁴ × Peak intensity (deg · cm ⁻¹ · M ⁻¹)
Poly(dA)	220	Positive	16
	250	Negative	8
	280	Positive	5
Poly(dT)	250	Negative	4
	280	Positive	2
Poly(dA).poly(dT)	220	Positive	8
	248	Negative	10
	255	Positive	3
	265	Negative	2
Poly(dA).2poly(dT)	280	Positive	1
	210	Negative	6
	250	Negative	8
	255	Positive	2
Poly(dA).2poly(dT)- Ψ -DNA	280	Positive	1
	210	Positive	50
	260	Positive	30
	290	Negative	20

found in the case of Ψ -DNA induced by spermidine, Co(NH₃)₆³⁺ and homospermidine, such a band was not present in the case of Ψ -DNA provoked by bis(ethyl)spermidine and other alkylated spermidines. The CD spectral changes observed in the presence of spermidine and its analogues are distinct from that reported previously for poly(dA) and poly(dT) in the single-stranded, duplex and triplex forms (summarized in Table 2) [15,34,35].

Among the compounds with bulky substituents, *N*⁴-cyclohexylspermidine allowed the largest concentration range (10–2000) over which *T*_{m1} and *T*_{m2} remained distinct. With *N*⁴-cyclohexyl- and 1,8-bis(ethyl)spermidines, conformational changes characteristic of the Ψ -DNA structure have not occurred in poly(dA).2poly(dT) until the polyamine concentrations reached 250 μ M. On the other hand, acetylation of the amino groups drastically reduced the efficacy of spermidine to provoke the triplex and/or Ψ -DNA forms. These results provide a rational basis for designing ligand structures capable of stabilizing triplex or Ψ -DNA structures over a predicted concentration range. These findings may further help in developing ligand-mediated triplex DNA formation as a novel therapeutic approach when specific genes with homopurine.homopyrimidine blocks can be identified [1–5].

Chemical structural effects of polyamines on their interaction with DNA and the resultant conformational alterations have been reported previously [8,18,19,36]. The differential effects of spermidine³⁺ and its substituted trivalent analogues on provoking and stabilizing unusual DNA conformations confirm the idea that the binding of polyamines with DNA is governed not only by ionic effects, but also by structural specificity effects, including steric constraints and charge separation. Using a series of putrescine and spermidine homologues, we previously reported

that the length of the methylene bridging region separating the amino and imino groups markedly influenced their ability to provoke Z-DNA and triplex DNA structures, whereas such structural alterations had a minimal effect on the stability of duplex DNA [8,19]. Our present study extends these previous observations and provides the first demonstration of the importance of steric factors in the binding of polyamines with DNA. Our results also show significant differences in the ability of polyamine analogues to provoke conformational transitions in triplex DNA, as determined by the efficacy of Co(NH₃)₆³⁺, spermidine³⁺, and spermidine analogues to exert an optimum stabilizing effect of the Ψ -DNA form (Table 1). The structural effects observed in this study may reflect relative affinities of polyamine analogues for DNA duplex and triplex forms. These results are in conformity with ²³Na relaxation studies showing differential affinities of 1,3-diaminopropane, putrescine, 1,5-diaminopentane and 1,6-diaminohexane for duplex DNA [37], although similar studies have not as yet been undertaken for the binding of polyamines with triplex DNA.

Our data also reveal a remarkable plasticity of the conformation of triplex DNA. Conformational alterations within the B-DNA and Z-DNA families have been known for some time [11–13,38]; however, a concerted effort to investigate the structure of triplex DNA has begun only recently. Triplex DNA may belong to the B-DNA conformational family, with a characteristic negative peak centred around 210 nm in CD spectral measurements. A ligand-induced conformational alteration was also reported with an inducible peak in the spectrum of poly(dA).2poly(dT) triplex at 300 nm [16]. Interestingly, the CD spectrum of (dG₃T₄G₃).d(G₃A₄G₃).d(C₃T₄C₃) also showed a strong positive band at 260 nm and a strong negative band at 277 nm [39]. In the present study, we found an inducible peak in the CD spectrum of poly(dA).2poly(dT) between 250 and 280 nm, and a negative peak at 295 nm. The polyamine concentrations at which maximal positive molar ellipticity occurred were also dependent on the ligand structure and the nature and positioning of the substituent. The efficacy of spermidine in inducing these structural modifications in DNA decreased with the number and bulkiness of the substituents on amino and imino groups. Although bulky substituents on pendant amino groups reduced the effectiveness of the analogues in provoking the triplex structure, this modification increased the concentration range over which triplex and Ψ -DNA structures were stabilized. The physiological and therapeutic implications of these results are currently under investigation in our laboratory.

The conformational class of triplex DNA with inducible positive and negative peaks at 260 and 295 nm, respectively, is not clear at present. The general features of the positive band are similar to those of Ψ -DNA, first described as a polymer and salt-induced transition [29]. Later studies have shown that similar transitions could be provoked in the presence of metal ions and polyamines [21,31]. However, the Co(NH₃)₆³⁺-induced Ψ -DNA in the duplex form of poly(dA-dT).poly(dA-dT) occurred at much higher concentrations (250–400 μ M) [31]. Furthermore, the positive band in this case was centred around 280 nm. Ψ -DNA in the poly(dA-dT).poly(dA-dT) duplex could produce either a positive or a negative band depending on the salt conditions. It is likely that the Ψ -DNA forms produced by triplex and duplex DNA might have different packing arrangements and chiral properties [21]. The remarkable structural specificity of spermidine analogues in imparting this conformation suggests a specific pathway in the wrapping of polyamine molecules over the DNA rather than non-specific associations. In other studies, we found that putrescine could not induce the Ψ -DNA conformation of poly(dA).2poly(dT), whereas spermine

was more effective than spermidine in inducing this transition (results not shown). Our results are consistent with the importance of the number of positive charges on polyamines in stabilizing Ψ -DNA and other unusual DNA structures.

Ψ -DNA is believed to be a tightly packaged, twisted, liquid crystalline form of DNA [28,40–42]. Ψ -DNA has been detected using certain combinations of DNA sequences and counter ions mainly by CD spectroscopy, although small-angle X-ray scattering and electron microscopic studies have also been reported. The CD spectrum of Ψ -DNA arises from the interaction of circularly polarized light with the highly ordered tertiary structure which depends on the proximity of the adjacent superhelical turns [41]. Recent studies have indicated a supercoiling-regulated liquid crystalline packaging of topologically constrained DNA [43]. The packaging buffer consisted of dehydrating conditions (0.8 M NaCl and 35 % ethanol). Electron microscopy of supercoiled plasmid DNAs under these conditions revealed elongated, tightly packed rod-like structures, in which striated texture that is either parallel or perpendicular to the main rod axis could be found. It is possible, but not yet proven, that Ψ -DNA may consist of such structures. Sharp crystal reflexes of type I Ψ -DNA formed from calf thymus DNA–spermine complexes suggested a two-dimensional DNA layer with constant DNA–DNA short periods between neighbouring molecules [42]. A type II form of Ψ -DNA formed from the same DNA/polyamine combinations suggested the existence of fluid-like or amorphous-like short-range disorder between neighbouring DNA molecules. Results presented in this study and previous studies on polyamine-induced condensation of DNA support the notion that these higher-order structures might be important in packaging of DNA in the cell [44].

The effect of chemical modifications of spermidine structure on its ability to stabilize triplex and duplex forms of poly(dA)–2poly(dT), as well as the ability of spermidine analogues to alter the conformation of triplex DNA, provide important new information on the mechanism of polyamine–DNA interactions. Although polyamine-mediated stabilization of duplex DNA has been known for more than three decades [45], the mechanism of polyamine–DNA interactions is not as yet clearly defined. Electrostatic, major groove-binding and minor groove-binding modes of DNA recognition of polyamine have been suggested by several investigators [19,20]. Single-crystal X-ray crystallography of oligonucleotide–spermine complexes showed a complex mode of interaction involving the binding of the amino groups of spermine in the major groove of DNA [46]. At low temperatures, however, X-ray diffraction analysis of oligonucleotide–polyamine complexes revealed minor groove binding of polyamines with DNA [47]. Photoaffinity cleavage with polyaminobenzenediazonium salts showed strong minor groove and weak A,T preferences of the polyamines [48]. Since the major groove of triplex DNA is occupied by the triplex-forming oligonucleotide, the minor groove becomes the preferred site of interaction for polyamines. It is possible that polyamines might crawl within the minor groove due to individual NH_3^+ groups jumping between multiple equidistant and isoenergetic bidentate hydrogen-bonding sites.

The major recent impetus for triplex DNA research is the potential of using site-specific, triplex-forming oligonucleotides (TFOs) in an anti-gene strategy to suppress the transcription of disease-related genes. For example, Postel et al. [7] showed that the treatment of HeLa cells with an oligonucleotide capable of forming triplex DNA at the promoter region of *c-myc* proto-oncogene inhibited its expression without affecting the transcription of other genes. Grigoriev et al. [49] further showed that cross-linking of a psoralen-oligonucleotide conjugate targeted to

the promoter of the alpha subunit of the interleukin 2 receptor gene inhibited the transcription of reporter plasmids transfected in living cells. We recently found that selective utilization of a polyamine could enhance the gene inhibitory effect of a TFO on *c-myc* oncogene in MCF-7 breast cancer cells, whereas this TFO had no significant effect on the transcription of *c-fos* [5]. Our present results on the possibility of sequential conformational transitions of triplex DNA in the presence of increasing concentrations of polyamines may have important consequences for the ability of TFOs to inactivate gene expression. Our current results suggest that derivatization and modifications of natural polyamines might provide a wide variety of compounds from which therapeutically relevant compounds could be selected for further studies, depending on the effects of these compounds on the dynamics of DNA conformations.

In summary, the data presented here provide a remarkable structural specificity effect of trivalent polyamines on the induction and stabilization of triplex DNA. In addition to the positive charge density on the counter-ion and the length of the methylene bridging region separating the positive charges on a polyamine, substitutions on the amino and imino nitrogen as well as the nature of the substituents dramatically influence the stability and conformation of the triplex DNA. Our results demonstrate considerable plasticity in the conformation of triplex DNA, influenced by ligand structural effects. This result is consistent with the recent report of a previously unknown structural description, P-DNA, for a peptide DNA-stabilized triplex DNA, based on single-crystal X-ray crystallography at 2.5 Å resolution [22]. Potential application of these findings include the development of specific ligands to stabilize triplex DNA in order to realize its potential as an anti-gene strategy for suppressing the transcription of disease-related genes. Future developments in this area may have a major impact on the development of gene therapy approaches to cancer, AIDS and autoimmunity.

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