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DNA Double Helix Destabilizing Properties of Cyclobisintercaland Compounds and Competition with a Single Strand Binding Protein

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Abstract—The DNA helix destabilizing activity of a series of cyclobisintercaland compounds (CBIs) has been evaluated by measuring their ability to displace a ^{32}P-labelled oligonucleotide primer (17-mer) hybridized to the single stranded DNA of M13. This destabilizing activity appears to be strongly dependent on the cyclic structure (the linear acyclic references are inactive) and the size of the macrocycle; both features being known to determine the preferential binding of the compound to ssDNA. Interestingly, CBIs induced the dissociation of the duplex template in a concentration range (0.5–1 μM) close to that required for the destabilizing activity of single stranded DNA binding proteins (SSBs). Therefore competition experiments between CBIs and an SSB protein (*Eco* SSB) for binding to a single stranded oligonucleotide target (36-mer) have been performed through gel electrophoresis and nitrocellulose binding assays and strong inhibitory effects on the formation of the SSB:36-mer complex have been observed.

Introduction

Cyclobisintercalands (CBIs) are water-soluble macrocyclic structures constituted of two intercalative units bridged by polyammoniums chains; three members of this series, namely bisnaphthalene (BisN), bisacridine (BisA) and bisquinacridine (BisQ) differing by the size of the aromatic unit are represented on Figure 1, along with their acyclic monomeric counterparts (mononaphthalene MN, monoacridine MA, monoquinacridines $MQ_{1\text{-}2}$). These compounds which exist predominantly in tetracationic form at/or near physiological pH, display the ability to associate strongly with nucleotides by the interplay of electrostatic attraction with the phosphate groups and stacking interactions with the nucleobases.[1–3] It has been shown that upon binding, the two macrocyclic ligands BisN and BisA adopt a conformation providing a distance of ca. 7 Å between the two aromatic moieties, thus allowing π–π stacking with the anionic substrate.[4,5] Moreover, the BisA macrocycle exhibited a clear binding selectivity for single stranded (ss) regions of nucleic acids which offer greater accessibility to the nucleobases.[6–8] The preference of BisA for binding to ssDNA has been attributed to both the cyclic framework of the molecule which disfavors the insertion into double helix sequences and to the short distance between the two acridine units which prevents the bisintercalation.[8] Because of its capacity to discriminate ss sequences from double stranded (ds) sequences, macrocycle BisA is able to induce the premelting of a long double helix such as poly[d(A-T)]$_2$.[9] Since only few organic compounds exhibit such a behavior,[10] it appeared interesting to further investigate this property, stimulated by the crucial role of DNA opening processes in the initiation of key-step events such as replication, transcription and recombination. Another persuasive reason to push forward our studies was the similarity between the binding properties of BisA and that of a class of proteins, namely ssDNA binding proteins (SSBs). These proteins constitute a structurally heterogeneous group but share the basic property to bind with high affinity to ssDNA without apparent sequence specificity.[11,12] They destabilize native dsDNA without requiring energy, in contrast to helicases, and most of them show the ability to

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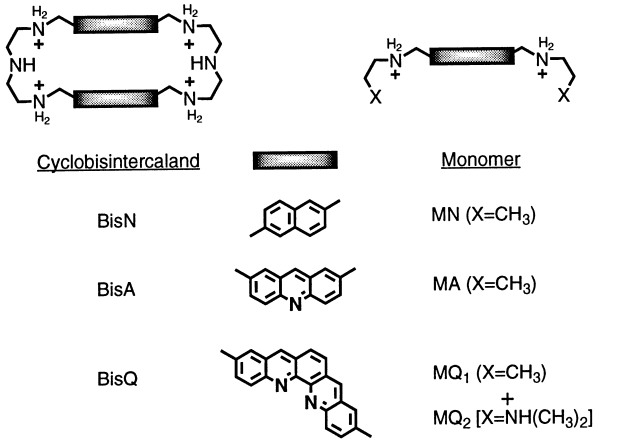

Figure 1. Schematic representation of CBI compounds and of their reference monomers at pH 6.0; N: naphthalene, A: acridine, Q: quinacridine.

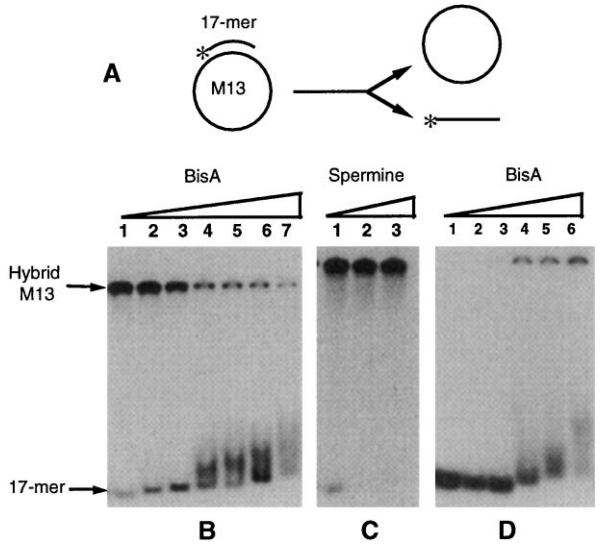

Figure 2. (A) Schematic representation of M13:17-mer hybrid. (B) Gel electrophoresis pattern of M13:17-mer hybrid alone (lane 1) and in presence of increasing amounts of BisA (lanes 2–7, [BisA] = 3, 6, 60, 90, 300, 600 μM). (C) Gel electrophoresis pattern of hybrid M13:17-mer in presence of spermine (lanes 1–3, [spermine] = 30, 90, 300 μM). [DNA] = 7 μM in phosphate unit; rt; cacodylate buffer (pH 6.0), 10 mM. (D) Gel electrophoresis pattern of oligonucleotide 17-mer alone (lane 1) and in presence of increasing amounts of BisA (lanes 2–6, [BisA] = 3, 6, 60, 90, 300 μM); [oligonucleotide] = 5 μM in phosphate unit; rt; cacodylate buffer (pH 6.0), 10 mM.

lower the melting temperature of synthetic polynucleotides.[13–17] SSBs are key proteins which act in concert with other DNA specific proteins (helicases, polymerases) involved in cell multiplication.[18,19] A number of them and their complexes with DNA have been extensively studied at the molecular level and amongst them, the *Escherichia coli* single-stranded binding protein (*Eco* SSB) provides an excellent prototype.[20,21]

On the basis of the DNA destabilizing property of BisA and in order to evaluate the analogy in the binding specificity of CBI compounds and SSBs, two types of experiments have been conducted. The first one was a simple assay that is commonly used to evaluate the helix-destabilization properties of SSBs;[14–17] it consisted in measuring the ability of CBIs to displace a ^{32}P-labelled oligonucleotide from its complementary sequence in the ssDNA of M13 (Fig. 2A). These displacement templates, initially designed for studying helicase activity,[22] have been widely developed as an alternative to the denaturation of long synthetic polymers since unwinding of natural duplex DNA is less widespread and more biologically relevant. They also provide a test with a sensitivity different from that of the melting of synthetic ds polynucleotides due to the presence besides the ds sequence of a ss tail that may favor the anchorage of the active protein.[15] This convenient test has been applied to compound BisA and to the homologous macrocycles BisN and BisQ, respectively, of smaller and larger size. The monomeric analogues of the CBIs, respectively MN, MA and $MQ_{1\text{-}2}$ already used as references in previous studies,[3,6] were tested in the present experiments as controls. In addition, spermine which bears an overall charge identical to that of the CBIs (4+) was used as polyamine control.

The second type of experiments was designed to compare CBI compounds and an SSB in terms of affinity for a specific single stranded DNA binding site. Therefore, competition experiments have been performed between CBI compounds and *Eco* SSB, which was used as a model for the binding to an ss oligonucleotidic target (36-mer). The goal was to determine if CBI compounds are able to inhibit the binding of the protein to ssDNA and eventually to interfere with its biological function.

Previously, in order to estimate the affinity of the various compounds for dsDNA relative to the ss conformation, thermal denaturation experiments of poly[d(A-T)]$_2$ were performed as with BisA.[9]

Results

Interaction of cyclobisintercalands with poly[d(A-T)]$_2$

Thermal denaturation experiments of poly[d(A-T)]$_2$ have been performed in presence of the two CBIs BisN and BisQ and of the reference monomeric compounds (MN, MA, $MQ_{1\text{-}2}$).

In presence of the reference monomers moderate to large increase of the melting temperature was induced without modification of the transition shape. The magnitude of the effect is very small in the case of MN whereas more significant ΔT_m values ranging from +8 to +15 °C were recorded with MA and MQ_1 (Table 1). The three compounds bearing the same cationic charge, these variations demonstrate clearly the dramatic influence of the aromatic surface area on the stabilization of the ds conformation. These results and in particular the effect of MQ_1 are in line with the high propensity of the quinacridines to stack with nucleobase pairs.[23] However, the larger increase in melting temperature induced by the tetraprotonated analogue MQ_2 emphasizes the significant additional contribution of electrostatics. $MQ_{1,2}$ can thus be assumed to intercalate between the

Table 1. Variation of the melting temperature (ΔT_m)[a] of poly[d(A-T)]$_2$ induced by the various compounds

Compound	ΔT_m (°C)
MN	+3
MA	+8
MQ_1	+15
MQ_2	+26
BisN	+8
BisA	−10[b]
BisQ	+15

[a]$\Delta T_m = T_m$ (complex)-T_m (free); [DNA] = 40 μM in phosphate units; [compound]/[DNA] = 0.15; cacodylate buffer (pH 6.0), 30 mM; NaCl 10 mM.
[b][Compound]/[DNA] = 0.2; see ref 9.

base pairs in a putative threading mode as has been described for similar compounds that possess ammonium linkers in opposite positions relative to the aromatic ring.[24] The stabilization due to MA, though moderate, might be as well attributable to the same type of interaction. In the case of MN, given the small amplitude of the variation, it is difficult to ascertain whether the effect is due to charge neutralization or insertion between the base pairs but this is certainly indicative of weak aromatic interactions.

By contrast to BisA, the two macrocycles BisN and BisQ stabilize the ds polynucleotide against thermal denaturation. However, the magnitude of the effect was weak in the case of BisN ($\Delta T_m = +8$ °C) whereas BisQ exhibited a stronger activity ($\Delta T_m = +15$ °C). Moreover, in the latter case enlargement of the melting profiles, significative of some distortion of the double helix and/or of some aggregation, were noted. The modest effect of BisN might be representative of a weak binding to the ds form resulting mainly from electrostatic interactions as with MN, which does not allow large shifts of the equilibrium. On the opposite, in the case of BisQ the interaction should be dominated by the contribution of the large aromatic units. Although the binding of BisQ should induce some local distortion from the normal double helix geometry as indicated by broadening of the curves, it results in a global stabilization of the double helix and thus a higher affinity for the ds conformation than for the ss form can be hypothesized.

Important precipitation of the DNA was observed in the case of the two macrocycles when the mixing ratio R (R = [compound]/[DNA] in phosphate units) was raised above 0.2. Precipitation of DNA by polyamines is well documented[25] but this phenomenon occurs at extremely low molar ratio with the macrocyclic polyamines and constitutes a major problem in the study of the interaction of the CBIs with nucleic acids as we previously reported.[9]

The interaction of the three CBIs with poly[d(A-T)]$_2$ is clearly influenced by the size of the aromatic unit and only BisA seems to possess the ability to destabilize the duplex form, thus exhibiting a clear binding preference for the ssDNA.

Displacement of a 17-mer oligonucleotide annealed to M13 ssDNA by cyclobisintercalands

On the basis of the above results the bisacridine macrocycle appeared to be the best candidate for destabilizing the hybrid system M13:17-mer and was tested first. The hybrid substrate (Fig. 2A) was incubated with increasing amounts of BisA in conditions that were chosen to favor the interaction (pH 6.0 where the cationic charge of the macrocycle is maximal and NaCl: 0 mM). The reaction products were then analyzed by electrophoresis on a native polyacrylamide gel, the large difference in mobility of the hybrid and the released oligomer allowing an easy detection of the two species. As the concentration in BisA was increased the progressive decrease of the non-migrating labelled material corresponding to the starting hybrid was observed together with the appearance of a fast migrating band corresponding to the free 17-mer (Fig. 2B). The beginning of the displacement was observed at quite low BisA concentration (lane 2), followed by a consistent dissociation (>75%) in the range 6–60 μM (lanes 3 and 4). A much larger excess of product was required to induce the almost complete disappearance of the hybrid substrate (>95%) (lane 7). Moreover a shift of the band of the released oligomer was observed when the concentration in BisA was increased above 60 μM. Spermine is totally inactive in the same conditions even at much higher concentrations (Fig. 2C). It seems that the macrocycle is able to displace the short oligomer from its hybridization site and furthermore to bind it once it is released in the medium. This trapping phenomenon, which seems to occur synchronously to the dissociation, could participate in the shift of the equilibrium;[9] this point will be discussed later.

However, the large excess of BisA needed to achieve a complete reaction raised some difficulties: firstly, enhanced ions concentration can induce local variations of pH and ionic strength and results in the observation of non specific effects, and secondly as noted above, precipitation of DNA was observed resulting in loss of a part of the labelled material stuck on the tube (see Fig. 2B, lane 7). Another difficulty due to charge neutralization is the inhibition of the migration in the gel of the complexes of various stoechiometries formed between BisA and the released 17-mer. Complexation of the free primer by BisA has been conducted in parallel and the analysis by gel electrophoresis is represented on Figure 2D. The labelled oligonucleotide is partially immobilized in the wells when the concentration in BisA is elevated (lanes 4–6) and this phenomenon can therefore interfere with the detection of the starting hybrid.

In an attempt to reduce the BisA concentration required for the complete 17-mer displacement, we tried to optimize the experimental conditions in varying parameters that affect the stability of the duplex such as temperature, salt concentration and pH. We thus performed melting temperature measurements of the hybrid in presence of the macrocycle at two different pH values (6 and 7) and at 0 and 100 mM NaCl. Practically, the hybrid was incubated at 25 °C and the temperature

raised by stages to +55 °C at which the complete dissociation was observed. Samples were taken every 5 °C, the reaction stopped as indicated in the experimental section and the results analyzed by gel electrophoresis. A typical experiment is represented on Figure 3A. The percentage of 17-mer released was quantified by densitometric scanning and plotted as a function of temperature to give a denaturation curve that allowed the measurement of a melting temperature with a satisfying accuracy (±5 °C). In these conditions, a destabilizing effect could be detected at low R ratio (R=[BisA]/[DNA]=1, $\Delta T_m = T_m$ (complex)$-T_m$ (free hybrid)= −5 °C) (Fig. 3B). This activity was moreover emphasized by further increase in pH to 7 ($\Delta T_m = -10$ °C; Fig. 3C) and in NaCl concentration to 100 mM ($\Delta T_m = -20$ °C; Fig. 3D). In this latter case, the denaturation curve of the complex was not shifted as compared to the preceding experiment (Fig. 3C) and the large difference between the two curves resulted mainly from the increased stability of the free duplex. The very low ionic strength dependence of the destabilizing effect of BisA characterizes a specific effect and suggests a relatively intimate interaction of the macrocycle with the base moieties and a lesser contribution of electrostatics with the negatively charged phosphate residues. Interestingly, the helix destabilizing activity of SSB proteins on similar systems has been described to be closely related to the salt concentration, in particular strong inhibitory effects have been observed when NaCl concentrations were raised above 20 mM.[13–15]

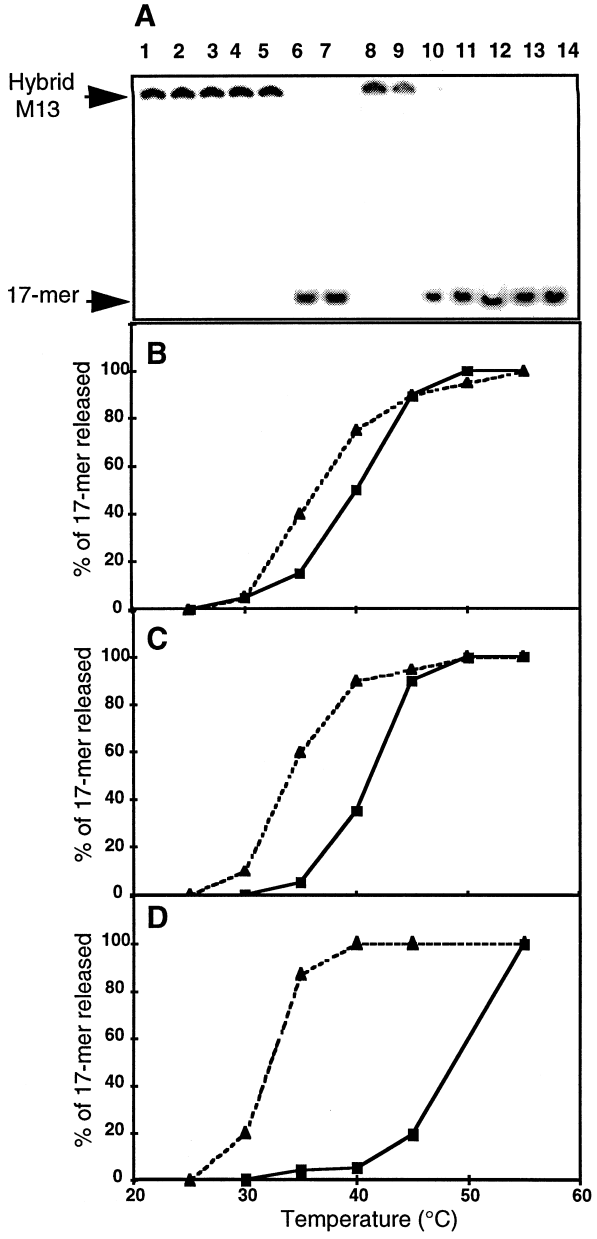

Figure 3. (A) Gel electrophoresis pattern after thermal denaturation of M13:17-mer hybrid alone (lanes 1–7) and in presence of BisA (lanes 8–14). Temperature was varied from 25 up to 55 °C by stages of 5 °C; Tris–HCl buffer (pH 7), 10 mM; NaCl 100 mM; [DNA] in phosphate unit = [BisA] = 1 μM. (B) Thermal denaturation curves of M13:17-mer hybrid alone (■) and in presence of BisA (▲) in cacodylate buffer (pH 6.0), 10 mM; NaCl 0 mM; (C) same in Tris–HCl buffer (pH 7.0), 10 mM; NaCl 0 mM; (D) same in Tris–HCl buffer (pH 7), 10 mM; NaCl 100 mM. [DNA] in phosphate unit = [BisA] = 1 μM in all experiments.

The thermal denaturation experiment was reproduced in the same conditions (pH 7.0, NaCl 100 mM, R=1:1) with the two other CBIs (BisN, BisQ), and with the control compounds (MA, MQ_2, spermine). The results are summarized in Figure 4. A significant destabilization was induced by BisN (Fig 4A; $\Delta T_m = -18$ °C) that was close to that induced by BisA (Fig. 3D; $\Delta T_m = -19$ °C). By contrast macrocycle BisQ exhibited a weak activity (Fig. 4A; $\Delta T_m = -3$ °C) that was similar to that of the reference compounds (Fig. 4B; in all cases $\Delta T_m < -5$ °C) which is hardly distinguishable from the experimental error.

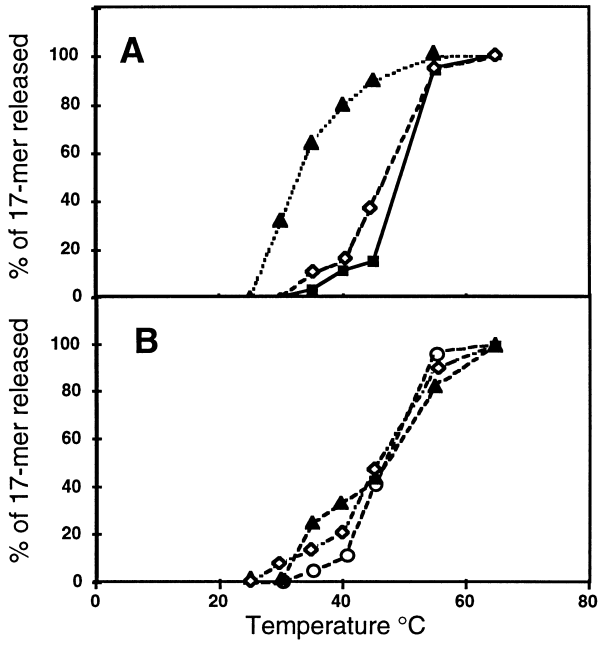

Figure 4. Thermal denaturation curves of M13:17-mer hybrid. (A) alone (■) and in presence of macrocycles BisN (▲), BisQ (◇); and (B) in presence of the reference compounds spermine (◇), MA (▲), MQ_2 (●); Tris–HCl buffer (pH 7.0), 10 mM; NaCl 100 mM, [DNA] in phosphate unit = [compound] = 1 μM.

To further assess the existence of two groups of compounds (i.e. active/nonactive), denaturation experiments have been performed as a function of the concentration in product. The results have been expressed by the proportion of 17-mer released at 40 °C as a function of the concentration in compound and are summarized on Figure 5. In the case of BisA and BisN, a clear concentration dependence of the activity was evidenced. However, it appeared from the curves that the maximum activity was not reached in the concentration range examined as there was no levelling off of the variation in both cases, but it was not possible to conduct experiments at higher [compound]/[DNA] ratio due to precipitation. Finally the slight variations induced in the case of BisQ and of the controls compounds were in accordance with the absence of a specific activity in the concentration range examined.

Competition between *E. coli* SSB and cyclobisintercalands for binding to a ss 36-mer oligonucleotide

To further investigate the analogy of behaviour between CBI compounds and SSBs, we examined the ability of BisA and BisN to compete with *Eco* SSB protein for binding to a ssDNA target. *Eco* SSB is one of the best characterized SSBs and its binding to oligonucleotides has been extensively studied. It exists as a homotetramer in solution and forms 1:1 complexes (1 mol tetramer: 1 mol oligonucleotide) with ss oligonucleotides of chain length of 30–60 residues.[19] We choose as a substrate a 36-mer oligonucleotide that was described to be tightly bound by the protein[26] and corresponds to the minimum site size.

Competitive binding experiments with this model system have been carried out in the presence of BisA and analyzed by gel retardation assay. Firstly, *Eco* SSB has been incubated with the 36-mer for 15 min at rt in a 1:1 molar ratio. The oligonucleotide concentration used was superior to the dissociation constant estimated for the protein: DNA complex (K_{app} >10^8 M^{-1})[19] thus ensuring that the free protein concentration was minimal in order to avoid interferences between the free species and the competitor in the medium. To this end, labelled and unlabelled (cold) 36-mer were mixed and the final oligonucleotide concentration was adjusted to 0.08 μM; these experimental conditions were indeed compatible with a total trapping of the oligonucleotide as shown by Figure 6A (lanes 1 and 2). The complex SSB:oligonucleotide was then incubated for further 15 min at rt with increasing amounts of BisA. As the concentration of BisA was enhanced, the band corresponding to the SSB:36-mer complex progressively disappeared and the labelled material was immobilized in the wells (lanes 3–7). The binding of 36-mer alone by BisA in the same concentration range has been carried out as a control experiment, and a similar pattern was observed: the band of the free DNA rapidly disappeared to give a species whose migration was inhibited (lanes 8–12). In the same conditions no shift of the complex was observed after incubation with large amounts of spermine (lanes 13–15).

These results might be interpreted in terms of competitive binding to the ss site, the macrocycle being able to inhibit the fixation of the protein to the DNA. In this hypothesis, the radioactive material in the wells is constituted by neutral or positively charged BisA:oligonucleotide complexes of various stoichiometries as seen from the control (lanes 8–12). However, the formation of a ternary complex between the oligonucleotide, the protein and the macrocycle involving non competitive interactions and whose mobility in the gel would be inhibited by charge neutralization could not

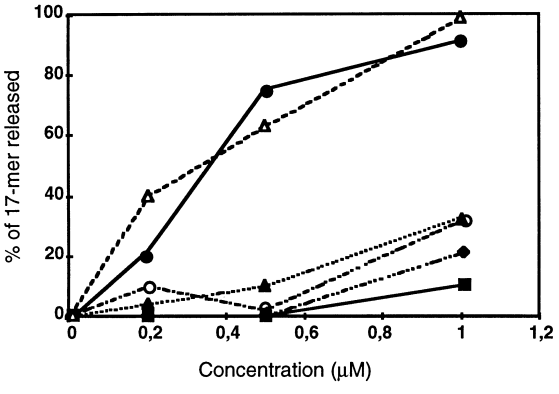

Figure 5. Fraction of 17-mer released at 40 °C as a function of concentration in compound BisA (△), BisN (●), BisQ (○), MA (■), MQ_2 (▲), spermine (◆) Tris–HCl buffer (pH 7.0), 10 mM; NaCl 100 mM.

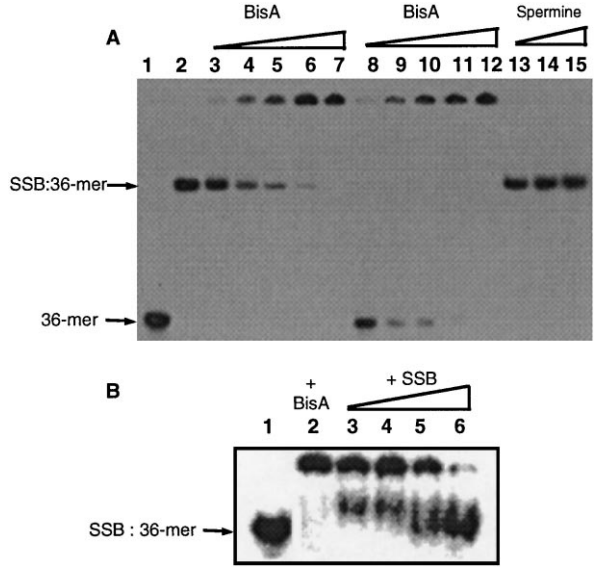

Figure 6. (A) Gel retardation assay of 36-mer oligonucleotide in presence of protein *Eco* SSB and of various compounds; lane 1: free oligonucleotide; lane 2: 36-mer + SSB; lanes 3–7: 36-mer + SSB + BisA (3, 7.5, 15, 30, 90 μM); lanes 8–12: 36-mer + BisA (3, 7.5, 15, 30, 90 μM); lanes 13–15: 36-mer + SSB + spermine (30, 90, 150 μM); [oligonucleotide] = [SSB] in tetramer = 0.08 μM; Tris–HCl (pH 7.0), 20 mM; EDTA 1 mM; DTT 1 mM; KCl 50 mM. (B) Gel electrophoresis pattern after displacement of complex (36-mer: BisA) by increasing amounts of protein *Eco* SSB. Lane 1: SSB 0.08 μM + 36 mer 0.08 μM; lane 2: lane 1 + BisA 90 μM; lanes 3–6: lane 2 + increasing amounts of SSB (3, 6, 9, 18 μM).

be completely ruled out. To test this possibility, the preceding competition experiment was reproduced and then the reaction mixture containing the 36-mer oligonucleotide, the protein SSB and the macrocycle BisA (Fig. 6B, lane 2) was incubated with increasing amounts of SSB. Figure 6B shows that as the concentration in SSB was increased, the spot in the wells diminished to the benefit of the band corresponding to the complex with the protein (lanes 3–6). This result demonstrates the existence of an equilibrium between the species immobilized in the wells and the SSB:36-mer complex and thus allows to exclude the presence of nonspecific ternary associations. However, ternary complexes in which both the protein and the macrocycle would be attached to the oligonucleotide in a competitive manner are likely to be formed transiently during the displacement reaction. Such intermediate species whose global charge would lie in between that of the two final binary complexes (SSB:36-mer and BisA:36-mer) could explain the shift of the band of the SSB:36-mer complex in the reverse displacement experiment of Figure 6B (compare lanes 3 and 6).

The two other macrocycles and the reference monomers have been tested in the same concentration range (3–90 μM). It appears that BisN has a behavior similar to that of BisA but is slightly less active, the total inhibition occuring at a higher concentration in macrocycle (90 μM versus 30 μM for BisA). BisQ elicited a weak effect even at the highest concentration examined (30–40% of inhibition at 90 μM) whereas MQ_2 and MA were unable to cause a significant variation of the migration of the SSB:36-mer complex (data not shown).

Finally, in order to overcome the difficuties raised by the gel electrophoresis method (i.e. the presence of non-migrating material) the interaction has been monitored by nitrocellulose binding assays. This method also provides a rough evaluation of the affinity of BisA for binding to the 36-mer oligonucleotide as compared to that of SSB. The SSB protein was immobilized on a nitrocellulose membrane and incubated with the labelled 36-mer in presence and in absence of BisA and of control compounds at increasing concentrations. The activity of the compounds were evaluated by quantification of the radioactivity retained on the membrane after washing. In the concentration range examined, the formation of the complex SSB:36-mer was inhibited by the macrocyclic BisA whereas spermine and the reference monomer MA elicited no effect (Fig. 7). Again this clearly demonstrates the inhibitory effect of BisA likely through a competitive binding to the oligonucleotide as was suggested by the electrophoresis gel shift assays. Moreover this allows to definitively rule out the formation of non-specific ternary associations between the macrocycle and the protein:oligonucleotide complex. Comparison with the inhibitory effect of unlabelled oligonucleotides 36-mer and 15-mer showed that the 15-mer and BisA have similar efficiencies to displace the labelled 36-mer from the protein binding site. This is clearly indicative of a high affinity of BisA for the targeted oligonucleotide (apparent binding constant of SSB:15-mer $>10^7\ M^{-1}$).[19]

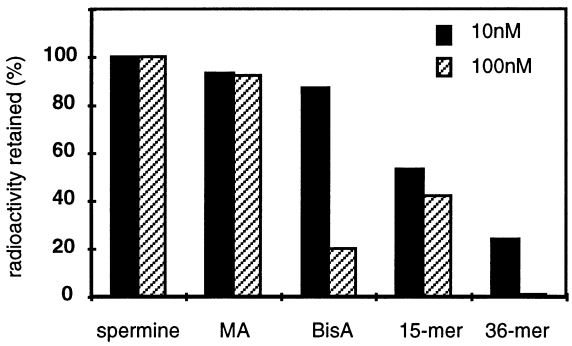

Figure 7. Nitrocellulose binding assay. Inhibition of the formation of complex (36-mer: SSB) by various competitors at two different concentrations (10 and 100 nM) in Tris–HCl buffer (pH 7.0), 20 mM; EDTA 1 mM; DTT 1 mM; KCl 50 mM.

Discussion

Amongst all the compounds tested, only the two CBIs BisA and BisN appeared able to dissociate the hybrid (M13:17-mer) substrate whereas the larger macrocycle BisQ and the linear monomeric references revealed inactive. These results are in part consistent with the effects observed with poly[d(A-T)]$_2$; as a matter of fact the most active compound is the macrocyclic Bisacridine that possesses a clear binding selectivity for the ss conformation whereas the macrocyclic Bisquinacridine which exhibited a high affinity for the ds polymer is inactive. This strongly suggests that the dissociation results from the ability of the macrocycle to form tight complexes with single strands, which is furthermore confirmed by the absence of effect of the linear reference monomers which associate to ssDNA with a lower affinity. The molar ratio CBI/DNA required to induce the destabilization of the substrate is identical to the protein/DNA ratio used with SSBs in similar conditions,[17] but whether the effect proceeds through a mechanism similar to that of the SSB proteins is more difficult to assess. The double helix destabilizing activity of SSB proteins usually proceeds by extensive coating of the single strand, due to favorable protein–protein interactions (cooperativity). It is probable that in the case of BisA there is a more or less statistical distribution of the ligand on the ssDNA template which, after reaching the saturation level, produces the displacement of the primer. However, there is no apparent reason for a cooperative binding but allosteric effects based on conformational unfolding of the ssDNA template could be envisaged. Moreover, and by contrast to the SSB behavior, the destabilizing effect of the macrocycle might also be related to the trapping of the oligonucleotide primer that could strongly help in shifting the equilibrium towards the dissociation as has been described with hairpins forming duplexes.[9]

Finally, the behavior of BisN which destabilizes the M13: 17-mer hybrid and stabilizes (though poorly) the poly[d(A-T)]$_2$ points to the structural differences between the two targeted systems as was mentioned in the introduction. Indeed some cases are known of SSB proteins that did not melt poly[d(A-T)]$_2$ while being

active in strand displacement with such M13 hybrid substrates.[15] This behavior is explained by the the presence of ss sites flanking the ds sequence, which may favor the anchorage of the protein. By analogy, and given the poor binding preference of BisN between ss and ds DNA, the fixation of the ligand should be preferentially directed on the numerous ss nucleotides present in large excess on the circular DNA and on the free 17-mer itself, thus shifting the equilibrium towards the dissociation.

The comparison of the effects of the three macrocycles which bear the same cationic charge but differ by the size of the aromatic unit, is very informative in terms of structure–activity relationships. The behavior of the macrocycles that likely stems from their ss versus ds binding preferences, is only weakly dependant on ionic strength and is therefore mainly influenced by the aromatic/aromatic interactions. The selectivities observed are in accordance with previous complexation studies that demonstrated that BisA and BisN were able to trap a single nucleotide[1,2] whereas BisQ was shown to accomodate a pair of nucleotides.[27] The peculiar complexation behavior of BisQ has been attributed to the strong tendency of quinacridines to stack with H-bonded associated bases such as base pairs or triplets[23] due to enhanced π-stacking interactions and a good structural complementarity. It is thus clear that the CBIs of small size such as BisA and BisN are more prone to stabilize exposed unpaired nucleic residues and that their binding selectivity is reflecting the balance existing between the electrostatic and the aromatic contributions in the interaction. In contrast, the binding of BisQ is strongly dominated by the strength of the quinacridine/base pair interaction, and in spite of the cyclic framework of the molecule there is a preferential fixation to a duplex sequence. Whether the fixation of BisQ occurs through a mono or a bisintercalation process is not known but it should certainly alter the normal geometry of the double helix causing a stable local rearrangement of the dsDNA as has been described for some natural antibiotics.[28] Similarly and by analogy with the SSB proteins that are known to extend ss polynucleotide backbone as a result of intrachain base unstacking, BisA and BisN binding may induce important conformational changes of the ssDNA. Indeed such effect is suggested by the significant increase in mobility on agarose gel of the ssM13 DNA in presence of BisA and BisN.[29]

Finally, the absence of effect of the monomers is in accordance with the low affinities that they display for single strands and supports that these compounds bind to ds conformations through a putative intercalative mode. Moreover, this result emphazises the structural requirement of a dimeric cyclic framework necessary to form a sandwich-like complex structure of high stability with the most accessible unpaired nucleobases.

The competition experiments with *Eco* SSB revealed that BisA was able to inhibit the fixation of the protein to the 36-mer oligonucleotide. This inhibitory effect seems to proceed through a competitive mechanism due to the high affinity of the macrocycle for the targeted ss oligonucleotide. The effects of the two other macrocycles are in line with their helix destabilizing abilities and thus with their respective affinities for ssDNA; BisN has a slightly weaker activity than BisA and BisQ is poorly active except at high concentration. Finally the failure of all reference compounds to inhibit the formation of the SSB:36-mer complex at any concentration tested, confirmed that the inhibition induced by macrocycles BisA and BisN is related to their specific molecular structure.

Conclusion

In summary, we have demonstrated that CBI compounds such as BisA and BisN display double helix destabilizing activities similar to single strand specific binding proteins. This property, which is strongly dependent on the size of the macrocycle, is likely to result from the ability of the macrocyclic structure to discriminate between single-stranded and double-stranded DNA conformations. The capacity of BisA and BisN to associate strongly to ss oligonucleotides leads to inhibit the formation of SSB protein:oligonucleotide complexes. Whether CBIs compounds are able to compete with other proteins for their ss binding site either on DNA or RNA is a subject of current investigation. Moreover the interaction of CBIs compounds with single stranded zones formed transiently during initiation of replication or transcription is a matter of much significance, either for designing agents able to interfere with polymerases activity or new tools for probing origins of replication.

Experimental

Reagents and buffer

All the solutions were made with distilled, deionized (MilliQ) and sterilized water. The synthesis of the CBI compounds BisA, BisN and BisQ and that of the reference monomers MN, MA, $MQ_{1\text{-}2}$ have already been described.[1–3]

DNA plasmid, oligonucleotides and protein

The oligonucleotides 17-mer 5′GTAAAAACGAC GGCCAGT and 36-mer 5′TACGCCAACAGCTCC TACGCCAACAGCTCCTACGCC were purchased from Genosys, stored and quantified as indicated by the supplier. Poly[d(A-T)]$_2$ was obtained from Sigma and the preparation of M13mp18 ssDNA was carried out as described.[30] *Eco* SSB was obtained from Sigma.

Thermal denaturation of Poly[d(A-T)]$_2$

The T_m measurements were performed with a Beckmann DU 640 spectrophotometer using constant heating rates of 0.5 °C/min and T_m values were calculated from the first derivative of the melting curves. Denaturation experiments were conducted in 30 mM

cacodylate buffer, 10 mM NaCl, with a typical DNA concentration of 40 μM (in phosphate units). Prior to the experiment all samples were heated in a water bath at 80 °C for 15 min and then slowly cooled to rt. The melting profiles were monitored at 260 and 270 nm and corrected for base line fluctuations.

Preparation of the M13:17-mer hybrid and unwinding assays

The 17-mer oligonucleotide was 5′-labelled with [γ-^{32}P] ATP and T4 polynucleotide kinase. M13mp18 DNA hybrid was formed by annealing the 5′-^{32}P-labelled 17-mer oligonucleotide to the complementary M13 ssDNA at a ratio 2/1 in 150 mM NaCl, 10 mM $MgCl_2$. After 5 min at 60 °C, the mixture was slowly cooled to room temperature and filtered through a spin column to remove the non-annealed oligonucleotide. Unwinding assays were performed in a final volume of 10 μL containing 10 mM cacodylate (pH 6.0) or Tris–HCl (pH 7.0) buffer, the hybrid DNA substrate (1–7 μM in phosphate unit) and the indicated amounts of CBI compound. Routinely, for the experiments carried out at room temperature, incubation time was 30 min. For experiments carried out at higher temperatures (T_m measurements) incubation was for 10 min, a sample was taken, the temperature raised and the procedure was repeated. To stop the reaction, each sample was treated with 5 μL of 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 30% glycerol and 0.5% SDS and placed at 4 °C. All the samples were then subjected to electrophoresis in a 8% polyacrylamide gel in 0.5× Tris-borate buffer. The gel was dried autoradiographed and the extent of the unwinding reaction was determined by densitometric scanning of the film. Each experiment was repeated at least three times.

Competition experiments with *Eco* SSB protein

Gel mobility shift assay. In a typical experiment, the protein (70 ng, 0.08 μM) was preincubated with the 5′-labelled 36-mer oligonucleotide (10 ng, 0.08 μM) for 15 min at rt in a buffer containing 1 mM EDTA, 1 mM DTT, 50 mM KCl and 10 mM Tris–HCl in a final volume of 10 μL. After preincubation, various amounts of compound (CBI or reference compound) were added to the reaction mixture and continuously incubated at rt for another 15 min. Prior to electrophoresis, 5 μL of loading buffer (0.1% bromophenol blue in 20% sucrose) was added to each sample, gel electrophoresis was carried out at rt in a 6% polyacrylamide gel in 0.25× Tris–borate buffer. The gel was dried and analyzed using phosphor-Imager (Packard) or densitometric scanning of the autoradiograph.

Nitrocellulose binding assay. 140 ng of SSB in 10 μL of PBS buffer was spotted on a nitrocellulose membrane, and preincubated for 1 h in 2 mL of binding buffer (1 mM EDTA, 1 mM DTT, 50 mM KCl and 10 mM Tris–HCl). The labelled oligonucleotide 36-mer (0.25 ng) and the competitor at the required concentration were added and incubation continued overnight. After washing several times with binding buffer, the radioactivity retained on the membrane was measured using phosphor-Imager.

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