Structural perturbation in supercoiled DNA: hypersensitivity to modification by a single-strand-selective chemical reagent conferred by inverted repeat sequences

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ABSTRACT

Bromoacetaldehyde, a reagent which modifies unpaired adenine residues, selectively modifies supercoiled DNA in the region of inverted repeats which are known targets for single-strand-specific nucleases. The reaction is dependent upon the topological state of the molecule, and the absolute importance of the inverted repeat has been demonstrated. Finer mapping of the distribution of the modification pattern reveals significant and interesting differences from the SI nuclease target positions. Bromoacetaldehyde modification is distributed over a wider region covering the whole inverted repeat, with greatest extent of reaction in the regions which flank the inverted repeat. It is suggested that an altered conformation may be propagated into these sequences. These results further support the contention that inverted repeats adopt an altered conformation when negatively supercoiled, for which the principal suggestion remains the cruciform structure.

INTRODUCTION

Many natural DNA molecules exist as torsionally underwound, or negatively supercoiled, species, and this has both structural and functional consequences. The free energy of supercoiling will drive processes which effectively reverse the underwinding, i.e., a local twist reduction may partially compensate for the linkage deficiency, and allow the molecule to adopt a more relaxed overall conformation. Perhaps the simplest example of this behaviour is the pre-melting of supercoiled DNA (1), and the high transcriptional efficiency of supercoiled templates (2,3) is another consequence. Even greater twist change occurs when a B to Z transition is undergone, and the stabilisation of local sections of Z-DNA by underwinding has been demonstrated (4,5). Thus there is both a strong theoretical case and considerable experimental support for supercoil-driven perturbation of DNA structure. Sequence-dependent structural polymorphism in DNA has become a subject of renewed interest with recent crystallographic (6,7) and solution (4,5,8-10) studies.

Negatively supercoiled circular DNA molecules possess topologically-generated single-stranded-like properties and are cleaved by single-strand-
specific nucleases such as SI nuclease (11). Examination of preferred sites of cleavage by these enzymes reveal that great site selectivity occurs (12-14) and mapping of these sites has shown each of them to possess inverted repeat sequence symmetry. Inverted repeats, or palindromes, have a formal conformational alternative, that of the intra-strand bonded hairpin loop or cruciform structure (15,16). This has been the most seriously considered explanation for the nuclease hypersensitivity data (12-14,17-20) for several reasons. Firstly, it provides a single-stranded loop region at the extreme of the stem-loop structure which might be expected to be susceptible to nuclease by a single-strand-specific nuclease, and such a view is supported by fine mapping of cleavages in the region (13). Secondly, cruciform structures are expected to be stabilised by negative supercoiling (2,12,14) since they provide a positive twist equivalent to melting the entire region, but at a fraction of the free energetic cost. Experimental observation and theory are once again in accord in terms of dependence upon both twist changes imparted (13) and linking deficiency (21,22, Otter, R., Lilley, O.M.J. and Cozzarelli, N., unpublished data). There remains, however, a serious reservation concerning the interpretation of data obtained by the use of single-strand-specific nucleases. In order to recognise single-stranded DNA, these enzymes must be capable of binding to it and thus they must possess single-strand stabilising protein-like activity. There exists a theoretical possibility, therefore, that in the absence of the enzyme the inverted repeats remain entirely in a regular helical conformation. Long inverted repeats produced in vitro have been shown to adopt cruciform geometry (23) when sufficiently negatively supercoiled, although for the particular sequences studied it appears that the activation energy involved in the interconversion process is relatively high (24). The question remains, therefore, whether or not the SI hypersensitive sites previously demonstrated in viable plasmids reflect cruciform structure which pre-exist in the torsionally stressed DNA. To attempt to answer this question a non-enzymic probe was sought, i.e. a single-strand-selective modification reagent. Such a species would have to be a small molecule for which there is no question of conformational stabilisation of the DNA. There are several documented examples of single-strand-specific chemical reactions (25-27) but the reagent chosen for the present investigation was bromoacetaldahyde, which has been developed as a probe of single-stranded DNA character by Kohwi-Shigematsu and Weintraub (28). The haloacetaldehydes (29) form an adduct with adenine residues at the hydrogen bonding nitrogen atoms and are thus ideally single-strand-selective. In this study it emerges that
inverted repeats confer considerable local hypersensitivity to modification by this reagent, but that the distribution of modification sites is different from the S1 cleavage loci. These results clearly provide further evidence for a pronounced sequence-dependent structural perturbation in DNA, and one which is dependent upon the topological state of the molecule.

MATERIALS AND METHODS

Plasmids

pColIR215 is a derivative of pBR322 where the region flanked by EcoRI and BamHI sites has been replaced by a 440bp fragment of CoIE1 DNA containing the inverted repeat which confers strong S1 nuclease hypersensitivity both on CoIE1 and pColIR215 (13).

pIRbke8 is a derivative of pAT153 containing a synthetic inverted repeat, and will be fully described elsewhere (30). In brief two 13 base oligonucleotides synthesised using phosphotriester methodology by Dr. A.F. Markham (Imperial Chemical Industries, Great Britain) were ligated to form a perfectly two-fold-symmetrical dimer, and cloned into the BamHI site of pAT153. The resulting plasmid is identical with pAT153 in every respect except the additional inverted repeat generated, the sequence of which is shown in Figure 2. Both plasmids were propagated in E.coli K12 M9101. Plasmid DNA was prepared by chloramphenicol amplification of log phase cells. After 16h cells were lysed using lysozyme, EDTA and Triton X-100 and supercoiled DNA isolated by isopycnic centrifugation in caesium chloride and ethidium bromide. Plasmid DNA was recovered by side puncture, butan-1-ol extraction and ethanol precipitation.

Bromoacetaldehyde

Bromoacetaldehyde was prepared according to Secrist et al. (29) and Kohwi-Shigematsu and Weintraub (28). Ten grams of bromoacetaldehyde diethylacetal were refluxed with 50 ml of 50% (w/v) sulphuric acid for 45 min, followed by distillation under reduced pressure. The distillate was adjusted to pH 4.5 using 1M sodium hydroxide and redistilled. The second distillate was approximately 1M bromoacetaldehyde.

Supercoiled DNA was reacted with bromoacetaldehyde in a buffer containing 50mM sodium acetate pH 4.5. Typically 2 μg plasmid was incubated for 30 min in a 50 μl total volume containing 100mM bromoacetaldehyde. Unreacted reagent was then removed by extraction into an organic phase, either buffered phenol or chloroform, isooamyl alcohol (24:1) mixture, followed by ethanol precipitation. Modified DNA was restriction cleaved under standard conditions.
After ethanol precipitation the now linearised DNA was digested with 8 units of SI nuclease in 30mM sodium acetate pH 4.6, 50mM sodium chloride and 1mM zinc chloride for 30 min at 37°C.

SI nuclease

SI nuclease from aspergillus oryzae was obtained from Bethesda Research Laboratories, and gave results which were indistinguishable with those obtained using SI nuclease purified locally using the method of Vogt (31).

Restriction Enzymes

Restriction enzymes were obtained from either New England Biolabs or Bethesda Research Laboratories, and used as directed by the manufacturer.

$[^{32}P]$ Kinase Labelling

DNA to be radioactively labelled with $^{32}P$ at 5'-termini was dephosphorylated by incubation with 15u of bacterial alkaline phosphatase (Bethesda Research Laboratories) at 65°C for 60 min in 20mM Tris pH 7.5, 0.1% SDS, followed by removal of enzyme using 20 µg/ml of proteinase K (Bethesda Research Labs) at 37°C for 30 min followed by phenol extraction and ethanol precipitation. Dephosphorylated DNA was $[^{32}P]$ labelled using 25u T4 poly-nucleotide kinase (New England Biolabs) with 10 to 50 µCi 2000 Ci/mMol $[^{32}P]$ATP (Amersham) in 50mM Tris pH 7.6, 10mM MgCl$_2$, 0.1mM EDTA and 5mM DTT. Alternatively, some marker fragments were exchange labelled (32).

Electrophoresis

1.3% agarose gels and 5-10% polyacrylamide gels were performed vertically using 90mM Tris pH 8.3, 90mM borate, 10mM EDTA for 16h. For autoradiography, polyacrylamide gels were dried on 3MM paper (Whatmen) and exposed to Kodak X-omat H film at -70°C using an Ilford fast tungstate intensifier screen.

RESULTS

Bromoacetaldehyde, like its chloro-analogue (29) may react at pH 4.5 with the nitrogen atoms of adenine bases to form a cyclic etheno derivative, shown in Figure 1, with recognised fluorescent properties. Since these two nitrogen atoms form the donor and acceptor atoms in the hydrogen bonded adenine-thymine base pair, they are only available for modification when the base is unpaired, and thus the reagent is single-strand-selective and may be used as a probe for 'melted' regions in DNA. In most of the experiments described here supercoiled DNA has acted as a substrate for bromoacetaldehyde modification, with the following scheme of sequential reactions used to identify regions which have been modified:
The supercoiled DNA was reacted with bromoacetaldehyde and the DNA purified;
(ii) The modified DNA was cleaved by a restriction enzyme to give a linear species;
(iii) The resulting DNA fragment(s) was cleaved by SI nuclease.
The rationale is that putative sites of hyper-reactivity towards the single-strand-selective reagent become modified and are therefore unable to re-pair, thus forming a 'bubble' of disrupted base-pairing. Following restriction cleavage which serves two purposes, to remove the torsional constraint and to provide a reference point for the subsequent cleavage, this perturbed region is cleaved by the single-strand-specific nuclease SI. Discrete fragments (i.e. narrow bands on gel electrophoresis) may only arise as a result of sequence-selective modification thereby conferring SI nuclease hypersensitivity to a specific region of the DNA molecule, which is necessarily in a fixed disposition with regard to the restriction site used.

**Hypermodification occurs at inverted repeats**

The plasmid pColIR215 [13], see Figure 2, was used in these studies since the ColEl inverted repeat that it contains is an extremely strong SI cleavage site when supercoiled. This sequence is, moreover, quite rich in adenine bases, the target for modification.

Figure 3 shows a result of pColIR215 reaction as a function of bromoacetaldehyde concentration, monitored by subsequent SalI and then SI cleavages. Whilst at the lowest bromoacetaldehyde concentration the predominant species observed is the full-length linear plasmid (L1), with higher concentrations of
Figure 2: Maps of plasmids used in these studies, pColIR215 and pIRbke8, showing relevant restriction sites and inverted repeats (filled boxes). The sequences of the inverted repeat regions are presented, with symmetric regions underscored. Sequence data for pColIR215 and the flanking region of pIRbke8 are taken from the data of Oka et al. (42) and Sutcliffe (43) respectively.

In order to map the modification site unambiguously it is necessary to repeat the reaction sets using other restriction cleavages, and examples are
Figure 3: Agarose gel electrophoresis of modification of pColIR215 as a function of bromoacetaldehyde concentration. pColIR215 was reacted with the indicated (5 to 500) millimolarity of bromoacetaldehyde, cleaved with Sall followed by SI nuclease. sc, supercoiled pColIR215; M, marker fragments obtained by HindIII restriction cleavage of phage PM2 DNA - sizes in base pairs indicated on right; S, position of native supercoiled plasmid migration; L1 and L2, migration position of full length and modified cleaved linear species respectively.

presented in Figure 4(a). In this experiment pColIR215 modified by bromoacetaldehyde has been cleaved with either BamHI, Sall or PstI before SI cleavage. From the known positions of these targets, see Figure 2, it can be deduced that the modification site lies approximately 100bp from EcoRI on the side distal to the replication origin. Subject to an experimental error of about ±20bp this region coincides with the position of the ColEl inverted repeat whose sequence is given in Figure 2, and which is responsible for the strong SI hypersensitivity of this plasmid. To emphasise this point directly analogous bromoacetaldehyde (B) and SI (S) experiments have been electrophoresed in consecutive tracks in the gel shown in Figure 4(b). Within the resolution of the gel system, the fragments which arise from bromoacetaldehyde modification and SI cleavage comigrate, indicating a common target region.

One difference between the two reactions, however, lies in their respective
Figure 4: Bromoacetaldehyde modification of pColIR215.

(a) Restriction mapping of modification position of pColIR215 using restriction cleavage by BamHI (B), SalI (S) and PstI (P). sc, supercoiled pColIR215; M, phage PM2 digested with HindIII.

(b) Importance of supercoiling, and comparison with S1 cleavage. L, SalI linearised DNA reacted with bromoacetaldehyde followed by S1; B, supercoiled plasmid reacted with bromoacetaldehyde, then cleaved by SalI followed by S1; S, supercoiled plasmid cleaved by S1 followed by SalI; sc, supercoiled pColIR215; M, phage PM2 digested with HindIII.

temperature dependences. Whilst the inverted repeat of ColEl and its derivatives may be S1 cleaved even at low temperatures (4°C), the bromoacetaldehyde reaction is negligible below 30°C.

A second point arises from Figure 4(b). Track L is the result of bromoacetaldehyde modification of linear pColIR215 DNA, as opposed to the previous studies on supercoiled DNA. This is achieved by reversing the sequence in which the bromoacetaldehyde and restriction endonucleolysis reactions are performed. No discrete bands are seen in this track, in contrast to those arising from S1 cleavage or bromoacetaldehyde modification of supercoiled plasmid. Clearly the localised modification at or around the inverted repeat sequence requires that the molecule be torsionally stressed, just as does S1
Figure 5: Bromoacetaldehyde modification of oIRbkeS and its parent plasmid pAT153. pIRbkeS was reacted with bromoacetaldehyde followed by restriction cleavage with SalI (S) or HindIII (H). pAT153 (pAT) was analogously treated as in (H). sc, supercoiled pIRbkeS; M, phage PM2 digested by HindIII.

cleavage at the same sequence (12,14).

Analogous experiments have been performed on a second plasmid, pIRbke8, see Figure 2. This plasmid contains a synthetic inverted repeat which acts as a strong S1 site (30), although not as strong as that of pColIR215. Like that of pColIR215 it is rich in adenine residues and might therefore be susceptible to bromoacetaldehyde modification. The results of these experiments are presented in Figure 5 and it is clear that just as with pColIR215 discrete bands are produced after modification, restriction cleavage and S1 nucleolysls reactions. Measurement of fragment lengths localises the position of hyper-modification to the region of the inverted repeat of this molecule. This site is, however, modified less readily than that of pColIR215, requiring greater bromoacetaldehyde concentration or longer reaction times, and in general producing weaker bands superimposed on a greater smear arising from non-selective reaction. In general terms, however, these two inverted repeats are evidently exhibiting analogous hypersensitivity towards the single-strand-
Figure 6: Finer mapping of modification - cleavage pattern in pColIR215.

(a) Comparison of SI and bromoacetaldehyde targets. Autoradiograph of 5% polyacrylamide gel electrophoresis of [5'-32P] labelled -340bp fragments arising from SI, BamHI cleavage (S) or bromoacetaldehyde (25mM), BamHI, SI cleavage (B). M, phage φX174RF DNA digested by HinfI and exchange labelled with [32P] as marker fragments.

(b) Modification pattern as a function of bromoacetaldehyde concentration. M and S as in (a); other tracks, modification by indicated millimolarity of bromoacetaldehyde.

The unique importance of the inverted repeat itself is demonstrable by the direct comparison of results of bromoacetaldehyde modification experiments on pIRbke8 and its parent plasmid pAT153 [33]. Figure 5 shows that the specific fragments observed with pIRbke8 have no counterpart in the case of pAT153. Since the only difference between the two plasmids is the introduction of the inverted repeat it is necessarily concluded that this feature, and this alone, confers the hypersensitivity to bromoacetaldehyde (and also to SI nuclease).

Distribution of modification

In order to examine the distribution of bromoacetaldehyde modification in
Figure 7: Finer mapping of modification - cleavage pattern in pIRbke8.

(a) Autoradiograph of 5% polyacrylamide gel electrophoresis of \([5'-32p]\)

labelled -360bp fragments arising from S1, HindIII cleavage (S) or bromo-
acetaldehyde, HindIII, S1 cleavage (B). M, phage \(\phi X174RF\) digested by
HinfI.

(b) Densitometer scan of tracks from (a).

greater detail, experiments were performed which were equivalent to those
above, but shorter DNA fragments were examined by kinase labelling 5'-termi

ni with \(^{32p}\) and autoradiography of polyacrylamide gels. This increases the
resolving power of the technique to 5-10bp.

Figure 6 shows the results of fine mapping the modification sites of
pColIR215, and a comparison with the analogous S1 cleavage experiment. One
point is striking - the two procedures do not result in identical fragments
being produced; the principal band seen after bromoacetaldehyde modification
is about 25bp shorter than the corresponding S1 fragment. With milder bromo-
acetaldehyde reaction radioactivity can be detected over 50bp, with peaks at
20-25bp on either side of the central S1 hypersensitive site. With greater
bromoacetaldehyde concentrations only the shorter fragments are detected, the
lengths of which become progressively smaller. Clearly a defect is present in
this approach in that the modified site is recognised by S1 cleavage; thus the
shorter fragments will be over-emphasised. This is seen directly by using an
alternative restriction site, such as the EcoRI target, which allows the obser-
vation of fragments arising from the other side of the inverted repeat.
The overall distribution of modification can be demonstrated more clearly in the case of pIRbke8, where levels of modification are generally lower. Figure 7 shows a polyacrylamide gel analysis of the SI and bromoacetaldehyde fragments arising from HindIII cleavage. It is seen very clearly that there is a broad distribution of radioactivity flanking the SI position, with peaks at approximately 20bp on either side. Densitometry of this distribution shows that the modification extends throughout the region, peaking at either extreme and the centre.

Taken together these results show that the entire local region of inverted repeats may become hypersensitive to bromoacetaldehyde modification when supercoiled, and that the hypersensitivity is actually greatest in the flanking region. And yet from the pIRbke8 - pAT153 comparison it is clear that the inverted repeat is solely responsible for conferring the hypersensitivity, despite the fact that pAT153 sequences themselves become extensively modified in pIRbke8, i.e. the flanking sequences. There is some evidence that under some circumstances SI nuclease may itself recognise structural perturbation in the flanking sequences. Figure 8 shows a comparison of bromoacetaldehyde modification and SI cleavage of pColIR215, both at 37°C, with restriction cleavage by SalI. Weakly staining fragments (SF) may be seen flanking the main SI cleavage (SM), which comigrate with the broader fragments arising from
chemical modification. This effect is not readily reproducible and is only seen when higher temperatures are used for the SI reaction.

DISCUSSION

Inverted repeat sequences confer localised hypersensitivity to modification by the single-strand-selective reagent bromoacetaldehyde when the DNA is negatively supercoiled. This has been demonstrated for two unrelated inverted repeats, and the crucial role of the inverted repeat itself has been established.

Local structural perturbation at torsionally stressed inverted repeats has previously been revealed by selective cleavage by single-strand-specific nucleases; principally SI nuclease (12-14) but also T7 nuclease (14) and micrococcal nuclease (34,35). Some major structural perturbation which is supercoil-dependent is therefore indicated, and cruciform (hairpin loop) formation has been suggested to be responsible for the observed nuclease sensitivity. This perturbation has now been demonstrated to be recognised by a non-enzymic probe, bromoacetaldehyde, thereby removing a possible objection to conclusions based on enzymatic probes alone. Single-strand-specific nucleases mechanistically must proceed via an intermediate in which the enzyme is bound to single-stranded DNA, and may therefore stabilise this form. Thus it is conceivable that the Km of the enzyme may itself induce a structural transition in the DNA. Whilst this point is rather weaker for micrococcal nuclease, primarily a double-strand endonuclease, it is clearly untenable for a small molecule such as bromoacetaldehyde which must, therefore, be recognising some pre-existing structural feature. In view of the inverted symmetry which is of fundamental importance for the observation of these effects, a strong candidate for this feature must be cruciform structure.

Cruciform formation can account for the nuclease hypersensitivity and its topological dependence in a simple way, and it is difficult to conceive structures which can provide an alternative.

What, then, of the differences between the effects of SI nuclease and bromoacetaldehyde? These are likely to be partly a result of the different molecular size and mechanism of these probes, and also partly as a consequence of the different experimental approaches necessitated by each probe. Interactions between an enzyme and another macromolecule such as DNA are likely to be severely restricted by steric constraints. It is therefore rather easy to envision a cruciform - enzyme interaction being limited principally to the extreme ends of the structure, at the unpaired loop. Conversely, a small
molecule like bromoacetaldehyde is subject to much less constraint and could
diffuse to many parts of the structure. A broader distribution of modifi-
cation might thus be expected together with the observed higher backgrounds of
non-selective attack. There is one very important experimental distinction
which must be made between SI and bromoacetaldehyde however. The former must
obey essentially single hit statistics, since once a cleavage has been intro-
duced the molecule is no longer supercoiled. No such restriction applies to
bromoacetaldehyde modification; indeed the reverse situation may well apply in
that once a region has become partially modified then this perturbation may
itself facilitate greater local modification. This is borne out to some extent
by the progressive increase in extent of modification seen with pColIR215 as
the reagent concentration is raised. It may be observed in a different manner
by studying topoisomer migration on chloroquine gels as a function of extent
of modification. An expected decrease in mobility is seen, but no clear limit
reached (Lilley, D.M.J., unpublished data), consistent with the production of
a 'bubble' whose extent increases with the degree of modification. A dif-
iculty with the 'expanding bubble' explanation is the observation of both
hypersensitive ends of the pColIR215 inverted repeat when observed from one
side. To explain this result it becomes necessary to postulate that the
modified DNA is itself relatively resistant to SI nuclease, but that it may
nevertheless hypersensitise adjacent nucleotides. This is difficult to rec-
concile, however, with the central hypersensitivity observed for pIRbka8, and
the cleavage of flanking DNA by SI nuclease at 37°C. I tentatively suggest,
therefore, that these regions have an altered DNA structure which is somehow
propagated from a central cruciform over 10-15bp on each side.

Such effects are not without precedent. The junction between B- and Z-
conformations has been shown to have an unusual structure in that it is
sensitive to SI-nuclease (4) and recently a crystallographic study of an RNA-
DNA junction demonstrated that the A-type conformation may be propagated into
a proximal DNA section (36). Work is currently in progress in this laboratory
to find other single-strand-selective reagents in order to extend these
studies. Bromoacetaldehyde is chiefly limited to adduct formation with
adenine residues, although cytosines may react at lower pH (29). Clearly it
is very desirable to broaden the extent of this probing with reagents which
will modify other bases.

A further advantage of small molecule probes lies in the possibility that
they may be sterically capable of reacting with transient but constrained
species. Cruciform structures are likely to be dynamic short-lived structures
and the intermediates which lie along the reaction surface may present new targets for bromoacetaldehyde reaction. This may help to explain the overall distribution of modification produced by the compound.

These studies provide new evidence for supercoil-driven perturbation of DNA structure which is sequence-dependent. This is likely to have functional consequences, for example in recombination (37), and a role has recently been suggested in the structure of the upstream domain of eukaryotic genes (38). Alternatively, these properties may confer undesirable consequences upon functional DNA molecules (39-41), and may therefore be features to be avoided. Whatever the actual operational significance, however, it is important to gain a better understanding of the structure and dynamics of these elements.

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