Activation of DNA Cleavage by Dynemicin A in a B–Z Conformational Junction†

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ABSTRACT: We report here that the DNA strand scission by dynemicin A is not only sequence-specific but also conformation-specific. The salt-induced B → Z conformational transition dramatically enhanced the cleavage by dynemicin A in a B–Z junction region. By contrast, the bleomycin–Fe(II) complex, the elsamicin A–Fe(II) complex, and esperamicin A_1 did not induce any preferential DNA cutting in such a DNA structure. The characteristic hyperreactivity of dynemicin A is observed in $(dC\text{-}dG)_8$- and $(dC\text{-}dG)_{12}$-inserted DNAs, but not in $(dC\text{-}dG)_5$-inserted DNA. These results suggest value in the use of dynemicin A as proof of the existence of a B–Z junction in vivo and also may aid in understanding the structure of B–Z junctions.

With respect to antitumor antibiotics targeted DNAs, their binding modes and cleavage mechanisms have been investigated actively (Goldberg, 1987; Zein et al., 1988; Kuwahara & Sugiura, 1988; Sugiura et al., 1989; Uesugi et al., 1991). Abnormal DNA as well as normal DNA is known to be a target of the antibiotics. For instance, neocarzinostatin chromophore and bleomycin caused specific scission at the site near a bulge structure of DNA (Williams & Goldberg, 1988). Bulged, curved, and junctioned DNAs appear to play an important role in various cellular processes. Unusual DNA structures may promote the insertion of such drugs between two adjacent DNA base pairs.

Dynemicin A (Figure 1) isolated from *Micromonospora chersina* is characterized as a hybrid molecule of two typical chemotypes of antitumor agents, namely enediyne and anthraquinone (Konishi et al., 1989, 1990). Recently, we indicated that the antibiotic binds to the minor groove of the DNA double helix and that its anthraquinone core probably intercalates into the DNA followed by attack of the phenylene diradical formed from the enediyne core (Sugiura et al., 1990).

We report here that DNA strand scission by dynemicin A is significantly activated at a B–Z junction of DNA. In pBR Z16 and Z24 DNAs, indeed, the salt-induced B → Z conformational transition dramatically enhanced the cleavage frequency by dynemicin A at a specific site of the B–Z junction region. Therefore, we propose that dynemicin A can recognize the unusual structure located in the DNA helix.

MATERIALS AND METHODS

Recombinant Plasmids. The plasmid DNAs used in this study (Figure 2), pBR Z10, Z16, and Z24, were recombinant plasmids derived from pBR 322 in which $(dC\text{-}dG)_5$, $(dC\text{-}dG)_8$, and $(dC\text{-}dG)_{12}$ oligonucleotides were inserted into the *Eco*RI site, respectively.

Drugs and Chemicals. Dynemicin A, esperamicin A_1, and elsamicin A were kindly provided by Bristol-Meyers Squibb Research Institute (Tokyo, Japan). *Eco*RI and *Dde*I were obtained from Takara Shuzo (Kyoto, Japan) and New England Biolabs, respectively. NADPH was purchased from Sigma. All other chemicals used were of commercial reagent grade.

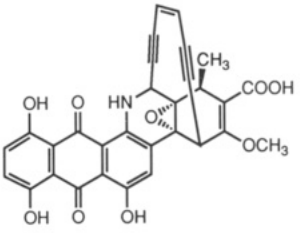

FIGURE 1: Chemical structure of dynemicin A.

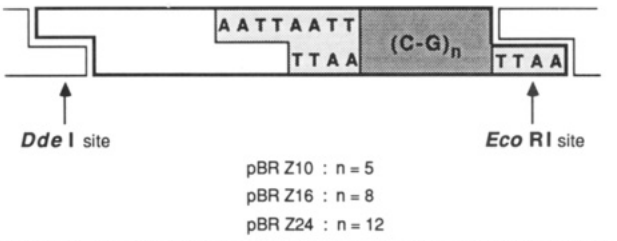

FIGURE 2: DNAs used in this study. These recombinant plasmids were digested by *Eco*RI, followed by *Dde*I.

Preparation and Labeling of DNA Restriction Fragments. The restriction *Eco*RI–*Dde*I fragment was labeled at the *Eco*RI site. The 5′-end was labeled by using polynucleotide kinase and [γ-^{32}P]ATP, after treatment with bacterial alkaline phosphatase (Maxam & Gilbert, 1980). The 3′-end was labeled with [α-^{32}P]dTTP by using the *Escherichia coli* DNA polymerase I large fragment. After digestion with the second enzyme, *Dde*I, the singly labeled fragment was purified by electrophoresis on a nondenaturing 5% acrylamide gel.

Nucleotide Sequence Analysis. The standard solution contains 20 mM Tris-HCl buffer (pH 7.5), 0–3.5 M NaCl, an excess of sonicated calf thymus carrier DNA (20 μg/mL), and a trace of the 5′- or 3′-end-labeled DNA. The mixture was preincubated at 37 °C for 14 h before the addition of dynemicin A (50 μM) and NADPH (50 mM). The cleavage reactions were performed at 37 °C for 8 h. Ice-cold ethanol and 0.3 M sodium acetate were added to the samples to stop the reactions, and the DNAs were recovered and desalted by repetitive ethanol precipitations. Electrophoresis was performed on a 10% polyacrylamide/7 M urea slab gel, and DNA sequencing was carried out by the Maxam–Gilbert method (Maxam & Gilbert, 1980). The autoradiograms were scanned with a laser densitometer (LKB Model 2222 Ultro-Scan XL).

Osmium Tetraoxide Reactions. Osmium tetraoxide (2 mM) and pyridine (2%) were added to the preincubated DNA

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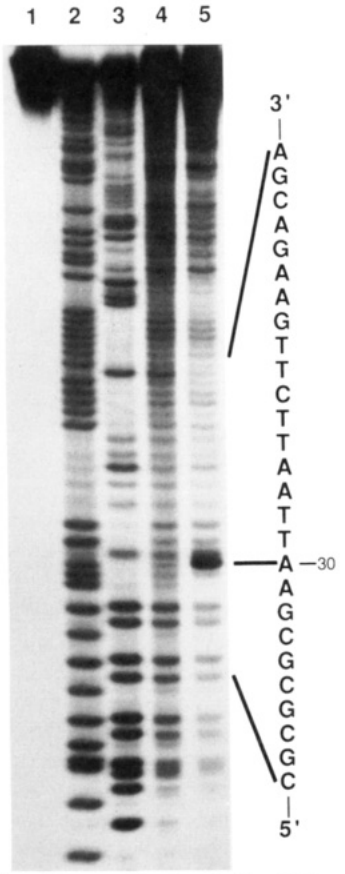

FIGURE 3: Autoradiogram of a denaturing 10% acrylamide gel of strand scission reactions. Lanes 4 and 5 show dynemicin A (50 μM)-induced DNA cleavage patterns in the absence and presence of 3.5 M NaCl, respectively. Lane 1 presents intact DNA. Lanes 2 and 3 show the Maxam–Gilbert sequencing reactions for A+G and C+T, respectively.

fragment in the standard solution, and then the reaction samples were incubated at 37 °C for 15 min. The reactions were stopped by extraction with chloroform and by precipitation with ethanol, and then the samples were lyophilized. The DNAs were cleaved at the sites of osmate adducts by treatment with 100 μL of 1 M piperidine at 90 °C for 30 min.

RESULTS

Cleavage Experiments of pBR Z10, Z16, and Z24 DNAs with Dynemicin A. Figure 3 shows the result of the cleavage experiments of pBR Z24 DNA with dynemicin A in the absence or presence of 3.5 M sodium chloride. As previously shown for normal DNA (Sugiura et al., 1990; Shiraki & Sugiura, 1990), dynemicin A also cleaved pBR Z24 DNA with relatively low sequence specificity in the absence of NaCl. Under the condition of the high salt concentration, interestingly, the cleavage intensity at the base site of A-30 was remarkably increased as compared with that under the salt-free condition. By contrast, the reactivity of the other base sites, in particular, cytosine bases within the $(dC\text{-}dG)_{12}$ block, was generally reduced. Figure 4 illustrates the densitometric tracings of the autoradiograms for the strand scission reactions performed in various salt concentrations. This dramatic enhancement of the cleavage intensity at the A-30 site near the $(dC\text{-}dG)_{12}$ block was observed in the concentrations of 2.5 and 3.5 M NaCl. In addition, the cleavage frequencies at the T-31, T-32, and A-33 sites under the condition of 3.5 M NaCl and at the T-31 site under the condition of 2.5 M NaCl were somewhat increased. The result indicates that the B–Z junction region may involve four base pairs.

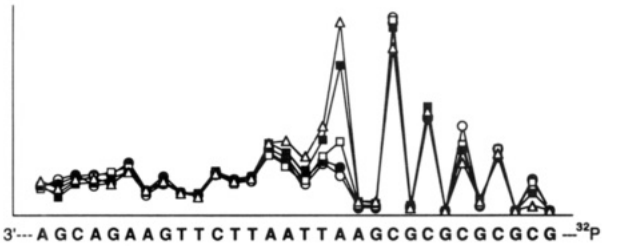

FIGURE 4: Densitometric analysis of DNA cleavage by dynemicin A. Relative cleavage frequencies of only the 30 bp containing $(dC\text{-}dG)_n$ block are illustrated. Open circle (○), closed circle (●), open square (□), closed square (■), and open triangle (△) represent the cutting patterns under NaCl concentrations of 0, 0.5, 1.5, 2.5, and 3.5 M, respectively.

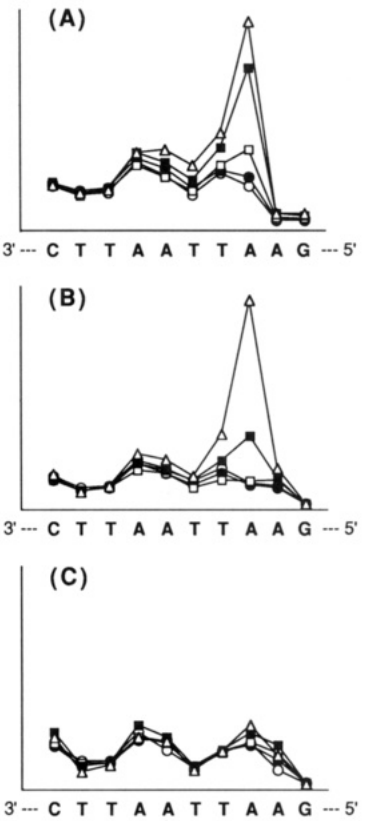

FIGURE 5: Densitometric analyses of Z24 (A), Z16 (B), and Z10 (C) DNA cleavages by dynemicin A. Only the neighboring region of the $(dC\text{-}dG)_n$ block is illustrated. Each symbol represents the same salt condition as given in Figure 4.

Figure 5 presents the results of the cleavage experiments by dynemicin A for three kinds of DNAs used in this study. When the concentration of the salt was increased, in the case of pBR Z16 and Z24 DNAs, the cleavage intensity by dynemicin A was evidently enhanced at the B–Z junction region, especially at the A-30 site. In contrast, no significant alterations of the junction region were detected in the case of pBR Z10 DNA. The reactivity of dynemicin A appears to be really sensitive to the formation of the B–Z junction.

Cutting Patterns of pBR Z24 DNA with Other DNA Cleaving Drugs. Under the experimental conditions of 0 and 3.5 M NaCl, similar DNA strand scissions by esperamicin A_1, the bleomycin–Fe(II) complex, and the elsamicin A–Fe(II) complex were investigated in pBR Z24 DNA (data not shown). As we previously reported, under the salt-free condition, esperamicin A_1 preferentially attacked T and C bases of oligopyrimidine sequences near the $(dC\text{-}dG)_{12}$ block. In the high concentration of the salt, the cleavage intensity at these sites was apparently reduced. Under the salt-free condition, the bleomycin–Fe(II) complex and the elsamicin

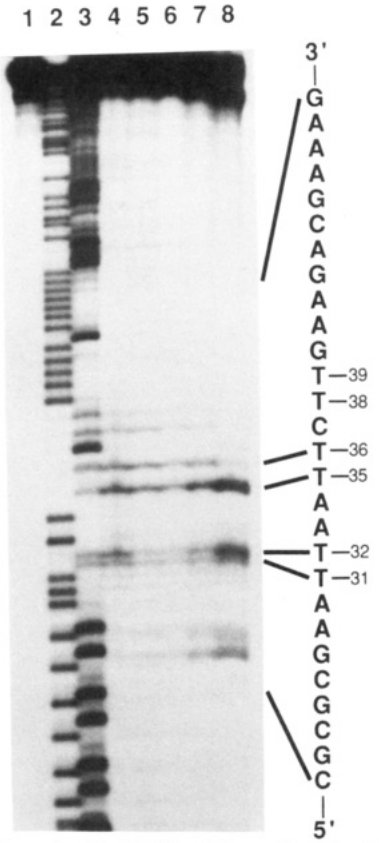

FIGURE 6: Polyacrylamide (12%) gel electrophoresis of OsO_4-reactive sites for the 5′-end-labeled DNAs. Under the salt concentrations of 0 (lane 4), 0.5 (lane 5), 1.5 (lane 6), 2.5 (lane 7), and 3.5 M (lane 8), the Os modification reactions were carried out at 37 °C for 15 min. Lane 1 presents intact DNA. Lanes 2 and 3 show the Maxam–Gilbert sequencing reactions for A+G and C+T, respectively.

A-Fe(II) complex showed little cutting for the present B–Z junction. In the high salt concentration, these drugs gave no prominent enhancements of DNA cutting in the B–Z junction region. The DNA cleavage result is clearly different from that of dynemicin A.

OsO_4 Modification. To examine the B → Z conformational transition of pBR Z24 DNA, we tried OsO_4/pyridine modification. The reagent OsO_4 is characterized as a pyrimidine-specific one that preferentially modifies such distorted regions of DNA caused by negative supercoiling as B–Z junctions and single-stranded sites in cruciforms (Galaska et al., 1986; Furlong et al., 1989).

Figure 6 demonstrates the OsO_4 modification of pBR Z24 DNA in the presence of pyridine. Under the condition of the salt (NaCl) concentration from 0 to 2.5 M, a pronounced alteration was not observed except for a minor change at the base sites of T-32 and T-35. In the 3.5 M concentration of NaCl, the cleavage intensity at T-32 and T-35 sites was strongly increased. In addition, the cuttings of T-36, T-38, and T-39 sites were almost disappeared. When the same reaction was performed at 4 °C, the reactivity of OsO_4 for pBR Z24 DNA in the NaCl concentrations from 0 to 2.5 M was negligibly weak compared to that in the presence of 3.5 M NaCl (data not shown). These results suggest that, under the condition of a lower NaCl concentration, the formation of a single-stranded DNA region may be caused not only by a B → Z conformational transition but also by "wobbling" and that some thymines in such a region are preferentially modified by OsO_4. Since the content of A/T bases is higher in the DNA region, the hydrogen bond formed is presumably weak. Therefore, the observed OsO_4 modification patterns under the condition of the low salt concentration may originate from weak base pairings. Further, guanine and cytosine bases located within the $(dC\text{-}dG)_{12}$ block were modified by OsO_4 in the presence of 3.5 M NaCl. Although a real reason for this behavior is unclear at the present stage, the conformation of the $(dC\text{-}dG)_{12}$ block in the high salt concentration probably differs from the conformation of normal B-form DNA. In normal B-form DNA, only thymine residues can be modified with OsO_4 (Friedmann & Brown, 1978). Under the condition of the high salt concentration, therefore, Z transition of the $(dC\text{-}dG)_{12}$ block is confirmed by susceptibility to Os modification and then B–Z junction is formed near the $(dC\text{-}dG)_{12}$ block region.

DISCUSSION

The cleavage hypersensitivity of dynemicin A was observed in pBR Z16 and Z24 DNAs under the condition of 3.5 M NaCl, but not in pBR Z10 DNA (Figure 5). Rahmouni and Wells (1989) indicated that a minimum insert length of $(dC\text{-}dG)_6$ is essential for stabilizing a Z helix. In addition, the midpoint of NaCl concentration for a B → Z conformational transition of DNA is estimated to be 3.6 and 3.25 M for the 157 base pair fragment obtained from a recombinant plasmid and a synthetic hexadecanucleotide, respectively (Klysik et al., 1981; Sheardy, 1988). Although the sequence of the B–Z junction investigated here is only one kind, the present result clearly shows that the salt-induced B → Z conformational transition activates the DNA cleavage by dynemicin A in the B–Z conformational junction. In the Z-DNA segment, the reduced reactivity of dynemicin A is consistent with the recent report (Guo et al., 1991).

We found that dynemicin A has potent cleavage activity for certain unusual structures of DNA. Indeed, the DNA-cleaving specificity of dynemicin A for an A/T-rich fragment is considerably higher than that for other DNA fragments (Shiraki & Sugiura, 1990). The A/T-rich sequences are known to have unusual DNA structure such as curved DNA (Trifonov & Ulanovsky, 1988). Although the DNA binding of dynemicin A is expected to be the intercalation of the anthraquinone core into DNA, the binding ability to normal B-form DNA appears to be lower than that of a typical intercalator such as ethidium bromide because of the steric hindrance of its enediyne moiety which is nearly perpendicular to the anthraquinone chromophore (Konishi et al., 1990). Therefore, a sufficient stereo-space is required for the stable binding of dynemicin A to DNA. Presumably, dynemicin A favors binding to abnormal DNAs. Bulged, curved, and B–Z-junctioned DNAs may satisfy such a situation, because these structures are found to induce unstacking or unwinding of the helix around the DNA regions (Woodson & Crothers, 1988; Nelson et al., 1987). We suppose that the cleaving ability of dynemicin A reported in this paper is closely related to its DNA binding affinity. A flexible region is necessary to a junction between two stable domains of DNA. Such a flexibility may facilitate the intercalation of dynemicin A with structural bulk. Recently, it was reported that ethidium unusually binds to deoxyoligonucleotide containing a B–Z junction (Suh et al., 1991). This result corresponds well with our finding.

Even at the concentration of 3.5 M NaCl, on the opposite DNA strand, no alterations of the cleavage frequency by dynemicin A were detected in the B–Z junction region (data not shown). An increase of the salt concentration did also not facilitate the OsO_4 modification for all thymines near the $(dC\text{-}dG)_n$ block on the opposite strand (data not shown). These

results suggest that the local conformation in the B–Z junction is asymmetric in both DNA strands. Since the enhancement of the cleavage frequency by dynemicin A is observed only at one or two bases on the unilateral strand (Figures 3 and 4), an interaction mode between dynemicin A and the present B–Z junction seems to be considerably unique. As reported previously, double-strand scission by dynemicin A was not detected in the B–Z junction site. Probably, the binding mode of dynemicin A to the B–Z-junctioned DNA differs from that to normal B-form DNA.

It has been reported that the bleomycin–Fe(II) complex causes specific scission at the site near a bulge (Williams & Goldberg, 1988). Recently, Guo et al. (1991) also observed the hyperreactivity of the bleomycin–Fe(II) complex for the Z-form segment of a DNA 16-mer. By contrast, the bleomycin–Fe(II) complex induced no preferential cleavages in the present B–Z junction. The results may indicate that the conformation of B–Z junction is unsuitable for minor groove binding of the bleomycin–Fe(II) complex. Clearly, OsO_4-modified T-bases existed in such a junction region. Accordingly, DNA helix distortion in the B–Z junction region is reliable.

On the other hand, esperamicin A_1 gave no preferred cutting sites in the B–Z junction region. It is unlikely that the enhanced cleavage in such a region by dynemicin A is predominantly due to the presence of the enediyne moiety. A recent dynemicin A model lacking the anthraquinone core cuts DNA less efficiently than dynemicin A (Nicolaou et al., 1991), suggesting that the DNA binding of dynemicin A is significantly influenced by the presence of the anthraquinone moiety. Probably, the DNA conformational specificity arises from DNA recognition by the overall structure of dynemicin A.

The existence of Z-form DNA is widely believed in vivo. The biological properties of the Z-region distinct from those of the other conformations of DNA might be essential for cellular processes such as recombination, transcription, and chromosomal events. The present hyperreactivity in the B–Z junction seems to be characteristic of dynemicin A combined with the enediyne core and the anthraquinone chromophore. Dynemicin A may be a useful reagent for probing some higher ordered structures of DNA which other drugs cannot detect. In addition, the bindings of carcinogens and mutagens appear to play an important role for stabilizing the Z-form DNA (Sage & Leng, 1980). Therefore, the property of dynemicin A demonstrated in this paper might be related to its antitumor activity. The present result shows that dynemicin A with relatively low sequence specificity of DNA cleavage attacks preferentially "one point" in a large DNA molecule under a particular condition. This study presents the first model for a dynamic targeting of antitumor antibiotics at the level of molecular biology.

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