Modulation of Mitomycin Cross-Linking by DNA Bending in the *Escherichia coli* CAP Protein–DNA Complex†

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ABSTRACT: We have examined the comparative reactivity of mitomycin cross-linking sites in DNA molecules either free in solution or complexed with *Escherichia coli* CAP protein. Sites in the region to which the protein is bound show strongly variable cross-linking by the drug. The reactivity of a CpG site located where the minor groove is narrowed by bending toward the protein was decreased by about 4-fold, compared to free DNA. The reactivity of a site placed so that the minor groove is widened by the bend was reduced by about 25%, and the reactivity of a $(CpG)_3$ sequence facing primarily away from the protein was reduced 25-fold by CAP binding. These results support the view that local DNA structure plays a critical role in determining the efficiency of cross-linking.

Previous studies in our laboratory (Teng et al., 1989) have shown a strong DNA sequence specificity of mitomycin cross-linking reactivity. By use of several compounds of the mitomycin class (NMA,[1] MS and MC) in reaction with different synthetic oligonucleotides, it was found that only the sequence CpG is susceptibile to cross-linking by the drug. Mitomycin enters from the minor groove of the double helix and covalently binds to the amino group of a guanine residue (Tomasz et al., 1987). When the adjacent upstream base is a cytosine, the $-NH_2$ group of the guanine on the opposite strand seems to be at an ideal distance to form another covalent bond with the drug, leading to a cross-link between the two strands.

The role played by the regions flanking the cross-linking site is not yet completely understood. An important aspect is the nonlinear relationship between the amount of cross-link and the number of (CpG)s when these sequences are adjacent to each other. In fact, mitomycins cross-link several (CpG)s better when they are in a row than when they are isolated (Teng et al., 1989). A variation in helical structure in runs of alternating CpG sequence is the probable source of this behavior.

A nucleic acid chain can undergo many different changes in secondary and tertiary structure during important phases of the life of the cell. It is therefore of importance to see if these different conformations are more or less favorable toward cross-linking by mitomycins.

A focus of study in our laboratory has been the CAP protein from *Escherichia coli*, a gene regulatory protein that binds as a dimer to DNA and stimulates transcription from a number of operons. The protein induces substantial DNA bending (Wu & Crothers, 1984), probably greater than 90° (Liu-Johnson et al., 1986). The binding/bending site is a region of about 36 bp (Liu-Johnson et al., 1986; Gartenberg & Crothers, 1988). The minor groove of the DNA binding site faces the protein at the approximate dyad axis (Majors, 1977; Simpson, 1980; Schmitz, 1981; Weber & Steitz, 1984; Waricker et al., 1987), and bending seems to be concentrated at the successive loci where major and minor grooves turn to face the protein (Gartenberg & Crothers, 1988). Since these properties are similar to those seen in bending of DNA by nucleosomes (Richmond et al., 1984; Satchwell et al., 1986), it is plausible to extrapolate from the altered mitomycin reactivity of DNA when CAP is bound, generalizing to the results that might be expected for DNA bound to nucleosomes or other proteins that induce bending. Thus our experiments address the general question of whether the changed topology of bent DNA in a protein complex can lead to alteration in the efficiency of cross-linking by the drug.

Materials and Methods

Oligonucleotides (Figure 1) were synthesized on an Applied Biosystems DNA synthesizer and purified on an 18% acrylamide gel, containing 7 M urea. They were ligated and cloned following standard procedures (Maniatis et al., 1982; Liu-Johnson et al., 1986). The CAP binding sites were constructed as described by Gartenberg and Crothers (1988). Fragments were labeled by primer extension with the Klenow fragment of DNA polymerase.

CAP was purified (Liu-Johnson et al., 1986) and stored in 400 mM KCl, 50% glycerol, and 50 mM phosphate buffer, pH 7.5. The protein was diluted to the desired concentration, just before use, with the following buffer: 50 mM KCl, 200 μg/mL BSA, 50% glycerol, 10 mM Tris, and 1 mM EDTA.

N-Methylmitomycin A, supplied by M. Egbertson and S. Danishefsky, was used as a 15 mM solution in DMSO and stored at −20 °C. Sixty millimolar sodium dithionite (Fisher) solutions were freshly prepared each time in deaerated water and used within 10 min.

Samples were prepared in 10 mM sodium phosphate (pH 7.5), 60 mM NaCl, and 0.1 mM cAMP solution containing the labeled DNA (≈10 000 cpm). The CAP–DNA complex was formed at room temperature by incubating for 10 min; 2 μL of 15 mM mitomycin was added, and the sample was deaerated by bubbling nitrogen for at least 1 min. Finally, 2 μL of 60 mM sodium dithionite was added. The final volume was 20 μL. The samples, tightly capped, were left on ice for 1 h (Cera et al., 1989). The reaction was then quenched with 5 μL of 5× loading dye (25% glycerol, 0.15% Bromophenol

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[1] Abbreviations: NMA, *N*-methylmitomycin A; MS, the aziridinomitosene of NMA (Danishefsky & Egbertson, 1986); MC, mitomycin C.

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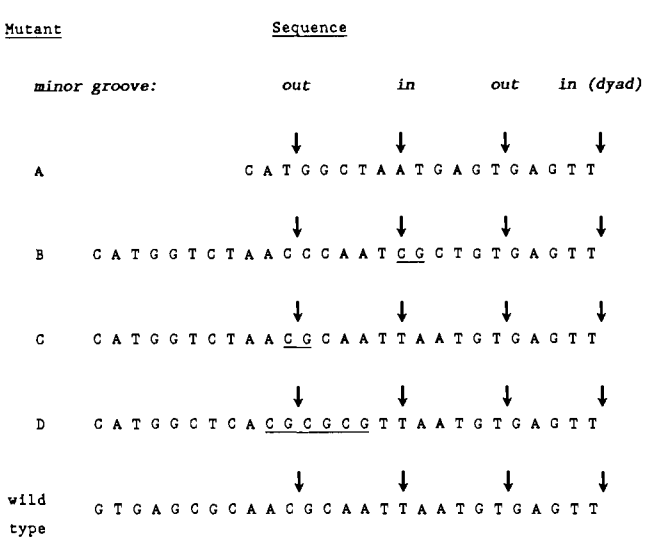

FIGURE 1: Sequences of the constructed CAP binding site: only the left side of the sequence, where the cross-linking targets are present, is shown. Reactive CpG sites are underlined. Arrows indicate the estimated orientation of the minor groove relative to the protein, either facing away from ("out") or toward ("in") the protein. When the minor groove faces the protein, it is narrowed by a roll bend, and it is widened when it is in the outward orientation (Gartenberg & Crothers, 1988). The sequence of the wild type is also shown for comparison.

Table I: Molar Ratios CAP/DNA Used and Percentages of Cross-Link

mutant	CAP/DNA[a]	% free DNA[b]	% cross-link -CAP[c]	% cross-link +CAP[d]	% cross-link in protein complex[e]	% cross-linking efficiency[f]
A	18	14				
B	24	30	1.8	0.8	0.4	24
C	12	16	2.6	2.0	1.9	73
D	18	20	8.6	2.1	0.4	4

[a] Molar ratio: the active concentration of the protein is considered as 30% of the actual concentration, as judged by DNA binding activity. [b] Percentage of free DNA when the protein is present. The value is an average of the results of several native acrylamide gels. [c] Values representing the percentage of cross-link in the absence of the protein. [d] Values derived from the denaturing gel, and representing the total amount of cross-link when CAP is present. [e] Values obtained from the following equation: [[% cross-link(+CAP)] - [% cross-link(-CAP)](fraction free DNA)]/(fraction bound DNA). [f] Numbers obtained from the following equation: [(% cross-link in complexed DNA)/(% cross-link in free DNA)](100) = % cross-linking efficiency.

Blue, 0.15% xylene cyanol, 5 mM EDTA, and 100 mM NaOH). The samples were boiled for 3 min and loaded on a denaturing polyacrylamide gel. When using a "double" gel (see below), the loading dye did not contain NaOH and the samples were not heated before loading. Other samples, containing DNA and protein, but not drug and reducing agent, were prepared following the same procedure. Control experiments (data not shown) were performed to assure that the conditions used for the cross-linking reaction affected neither the stability nor the electrophoretic mobility of the protein complex. The ratios of CAP/DNA used are reported in Table I.

Acrylamide gel electrophoresis was used to evaluate the results. On a 10% native gel in TBE buffer (89 mM Tris, 89 mM borate, and 2 mM EDTA) the CAP–DNA complex can be separated from the free DNA, but a 17% gel, containing 7 M urea, is necessary to separate the cross-linked DNA from the un-cross-linked product. We also used a modified "double" gel, composed of two sections: an upper 10% native gel and a lower 17% denaturing gel. On the first part the CAP–DNA complex is separated from the free DNA by running for 2 h at 12 W. Then 15 μL of 3% SDS is loaded into each well. Since SDS moves faster in the gel, it reaches the DNA and destroys the complex with the protein, before the nucleic acids enter the denaturing section of the gel. The DNA is denatured inside the gel by the heat generated by the current, running 2 h at 60 W. In the denaturing section the cross-linked product is separated from the un-cross-linked DNA. The use and importance of this technique will be explained under Results and Discussion.

The results were quantified by cutting the gel slices and counting them in a Packard scintillation counter.

Results and Discussion

Our experiments were designed to determine whether free DNA or DNA bound to CAP is more susceptible to cross-linking by mitomycin. We consider two main factors in interpreting our results: alterations in the geometry of the nucleic acid in the complex, and possible electrostatic or steric effects that might reduce the noncovalent binding of the drug which is a necessary prerequisite for cross-linking. We exclude any specific reaction between the drug and the protein that affects binding, as proven by control experiments (data not shown).

Several mutants of the CAP binding site were prepared, with CpG in different positions along the sequence (Figure 1). The DNA binding site for the protein has partial dyad symmetry, and on the right hand of the dyad axis no (CpG)s are present in the sequence. On the left side the wild type contains two (CpG)s, one of which is in a region not directly bound to the protein. This fact allowed us to build mutants with only one cross-linking site (mutants B–D) by modifying only the left side of the protein binding site. The control sequence, mutant A, lacks a reactive CpG site. All of the mutants were tested for their binding affinity for the protein. Titrations with CAP showed that the stability of the complex is not identical for all of them. Therefore, in order to have at least 70–80% of the nucleic acid bound to the protein, different ratios of CAP/DNA were used, as reported in Table I. However, even with different binding affinities, these mutants are bent approximately to the same extent, as indicated by their mobilities on a nondenaturing gel (Fried & Crothers, 1983).

Sodium dithionite and DMSO, used to dissolve the drug, affected neither the stability of the protein complex, nor the DNA itself (data not shown).

The technique of the "double" gel, reported under Materials and Methods, permitted a preliminary separation of DNA bound to the protein from noncomplexed DNA. These two bands were further split into two more bands each in the lower denaturing gel. Unfortunately, the bands are smeared, due probably to the incomplete denaturation of the samples inside the gel and to the partial retention of complex between DNA and protein. An example is reported in Figure 2. This assay did not allow an accurate quantitation of the results but is useful in verifying directly that cross-linked DNA was bound to CAP. It was used for this purpose, in parallel with a simple denaturing gel (an example is shown in Figure 3).

The same samples were divided in two aliquots and loaded respectively on a "double" gel and on a 17% denaturing gel. The former enabled us to confirm the formation of the protein complex and see if there was cross-linked DNA in it, while the latter contained sharp bands that could be cut out from the gel and counted in a scintillation counter. It should be recalled that the cross-linked product contains both complexed and free DNA; the amount of the latter was determined as the average of several titrations with the protein. Therefore, in evaluating the percentage of cross-link present only in the nucleic acid bound to CAP, the amount due to the free DNA

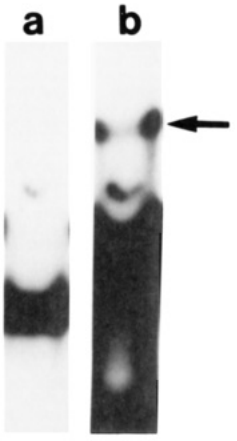

FIGURE 2: Example of a "double" gel (overexposed). The bands corresponding to free DNA are not reported. (a) DNA not treated with CAP but reacted only with mitomycin: the band corresponds to the cross-linked product. (b) DNA reacted with mitomycin after the formation of the DNA–protein complex: the arrow indicates the position of the cross-link present in the DNA complexed with the protein. The band of non-cross-linked DNA bound to the protein is largely smeared, for the reasons explained in the text. The faint small band present in both the lanes is due to SDS. The mutant used here is C, but the same result is obtained by using mutant B or D.

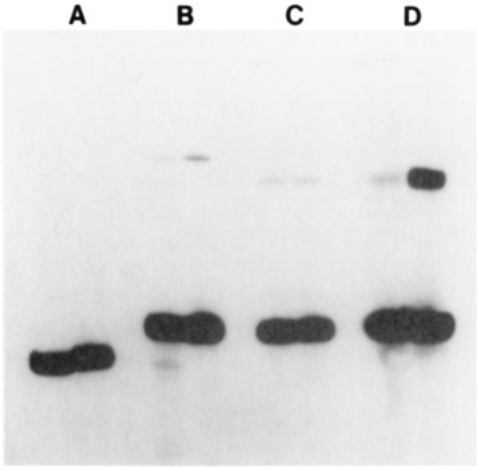

FIGURE 3: Acrylamide (17%) denaturing gel. The mutants are in the order A, B, C, and D. In each pair of lanes the right lane contains DNA not treated with CAP but reacted only with mitomycin, and the left lane contains DNA reacted with mitomycin after the formation of the DNA–protein complex. The cross-linked products (upper bands) have slightly different mobility on the gel, according to the different position of the cross-links in the sequence. It is evident that in mutant A no cross-link at all is present. Furthermore, mutant A has different mobility on the gel, reflecting the different length of the DNA, due to the deletion of 8 bp outside the binding site.

was calculated and subtracted from the total cross-linked product. This has been done for each mutant, and the average of several experiments is reported in Table I.

Again our data show that no cross-linking at all occurs if the sequence contains no (CpG)s (mutant A), confirming the previously deduced specificity of reaction of mitomycins (Teng et al., 1989). The presence of the protein affects the yield of the cross-linking reaction to a different extent in all the mutants examined. The perturbation of reactivity seen in mutant B, in which the minor groove at the CpG site faces inward toward the protein, is intermediate; in this case the complex is 24% as reactive as the free DNA. Steric exclusion and electrostatic repulsion of the drug would be expected to be maximal in this case. However, some cross-link is still expected, since both the major and the minor groove should not be completely protected by the protein when facing it: DNA wrapped around the histone core, a conformation close to the one we examined, is remarkably accessible to small molecules in both the major and the minor groove (McGhee & Felsenfeld, 1979). The smallest reduction in cross-linking is seen when a single reactive site faces away from the protein: mutant C is 73% as reactive in complex as in the free DNA. Cross-linking in mutant D is strongly reduced in the CAP–DNA complex, to 4% of its value in free DNA. The repeated CpG sites in this mutant lie predominantly on the outside of the protein–DNA complex, where the minor groove should be widened due to bending. Given the comparison with mutants B and C, and model building which shows no protein groups on that side of the DNA (Waricker et al., 1987), it is unlikely that the dramatic loss of reactivity of mutant D can be due to electrostatic repulsion or steric exclusion of the drug. Rather, we conclude that widening and distortion of the minor groove by bending at the reactive CpG site in mutant D leads to a reduction in cross-linking efficiency. DNA bending may also cause loss of the special conformation of repeated CpG segments which leads to high cross-linking efficiency (Teng et al., 1989). This reason might not be sufficient to explain the difference between mutants C and D, since the absolute amount of cross-link in the DNA complexed with the protein is lower for the latter (Table I). On the other hand, the distance between the guanine amino groups of the CpG sites is slightly different in the two mutants, and the flanking regions may play a key role, not yet completely understood.

The evident importance of geometric factors for mitomycin cross-linking is consistent with our interpretation of the preferential reactivity for CpG compared to GpC (Teng et al., 1989), in which it was found that the distance between the two guanine amino groups in GpC sequences is substantially greater than required for covalent bond formation. Widening the minor groove at a CpG site by introduction of a roll wedge between the two G·C pairs will lengthen the $-NH_2$ to $-NH_2$ distance from its near optimum in CpG and should result in a reduction in reactivity, as observed.

During the life of the cell, DNA is often bent or somehow distorted, as happens upon interaction with many proteins (histones, topoisomerases, regulatory proteins, etc.). The results of our experiments suggest that any distortion that widens the minor groove at a CpG site will reduce the efficiency of cross-linking by mitomycin. At present, the most likely candidates for highly reactive sites in cells are runs of alternating CpG sequence that are not complexed with protein.

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References

Cera, C., Egbertson, M., Teng, S., Crothers, D. M., & Danishefsky, S. J. (1989) *Biochemistry* (submitted for publication).

Danishefsky, S. J., & Egbertson, M. (1986) *J. Am. Chem. Soc. 108*, 4648–4650.

Fried, M. G., & Crothers, D. M. (1983) *Nucleic Acids Res. 11*, 141–158.

Gartenberg, M. R., & Crothers, D. M. (1988) *Nature 333*, 824–829.

Liu-Johnson, H., Gartenberg, M. R., & Crothers, D. M. (1986) *Cell 47*, 995–1005.

Majors, J. (1977) Thesis, Harvard University.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
McGhee, J. D., & Felsenfeld, G. (1979) *Biochemistry 76*, 2133–2137.
Schmitz, A. (1981) *Nucleic Acids Res. 9*, 272–292.
Simpson, R. B. (1980) *Nucleic Acids Res. 8*, 759–766.
Teng, S. P., Woodson, S. A., & Crothers, D. M. (1989) *Biochemistry* (preceding paper in this issue).
Tomasz, M., Lipman, R., Chowdary, D., Pawlak, J., Verdine, G., & Nakanishi, K. (1987) *Science 235*, 1204–1208.
Waricker, J., Engelman, B. P., & Steitz, T. A. (1987) *Proteins 2*, 283–289.
Weber, I. T., & Steitz, T. A. (1984) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3816–3820.
Wu, H. M., & Crothers, D. M. (1984) *Nature 308*, 509–513.

Effects of Aluminum and Other Cations on the Structure of Brain and Liver Chromatin†

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ABSTRACT: The reactivity of aluminum and several other divalent and trivalent metallic cations toward chromatin from rat brain and liver has been investigated. Two criteria are used to determine the relative reactivity of these cations toward chromatin. The first involves the ability of the ions to compact the chromatin fibers to the point where chromatin precipitates. The second criterion measures the ability of cations to interfere with the accessibility of exogenous structural probes (nucleases) to chromatin. Of the divalent cations tested, nickel, cobalt, zinc, cadmium, and mercury were the most reactive toward chromatin, on the basis of their ability to induce precipitation of chromatin in the micromolar concentration range. The divalent cations magnesium, calcium, copper, strontium, and barium were much less effective, although all cations precipitate chromatin if their concentration is increased. Of the trivalent cations tested, aluminum, indium, and gallium were very effective precipitants, whereas iron and scandium were without effect at the concentrations tested. Of all the cations tested, aluminum was the most reactive. Aluminum's ability to alter the structure of chromatin was investigated further by testing its ability to interfere with nuclease accessibility. This test confirmed that aluminum does induce considerable changes in chromatin structure at micromolar concentrations. Furthermore, chromatin from cortical areas of the brain was much more sensitive to aluminum than chromatin from liver. These results are discussed in light of the known toxicity of these cations, with particular emphasis on the possible role of aluminum in Alzheimer's disease.

The transcription of a gene into mRNA by RNA polymerase is preceded by a complex series of changes in the structure of DNA as it is unfolded from a compacted chromatin fiber in order to make the coding strand accessible to RNA polymerase. Divalent cations play vital roles in these reactions. For example, magnesium ions and probably calcium ions are involved in the stabilization of DNA in chromatin fibers (Eichhorn, 1979, 1981; Walker & Sikorska, 1987a,b), and calcium and copper ions appear to be involved in the stabilization of the highest levels of chromatin structure, the chromatin loop domains (Lebkowski & Laemmli, 1982). Furthermore, zinc ions together with a second divalent cation, typically manganese or magnesium, are essential for RNA polymerase activity (Mildvan & Loeb, 1981). Given such essential roles for divalent cations, the question arises of how other metallic cations, particularly those derived from the environment and believed to be toxic, might interfere with these reactions. This area has been poorly studied, but some earlier studies have indicated that nonphysiological cations can increase the error rate of RNA polymerase (Eichhorn, 1979; Loeb & Mildvan, 1980) and can interact with DNA (Record et al., 1978; Eichhorn, 1981).

Considerable information now exists on the effects of physiological cations on chromatin structure, and, indeed, monovalent and divalent cations are routinely used to manipulate the structure of chromatin in vitro in studies designed to elucidate the structure of the chromatin fibers (Walker & Sikorska, 1986, 1987a,b; Walker et al., 1986). Using this information, it is now practical to carry out a more meaningful examination of the effects of inorganic metal cations on the structure of chromatin. In this paper, we have examined the effects of a number of cations on chromatin from rat liver [for which the bulk of structural information is available (Walker & Sikorska, 1986, 1987a,b; Walker et al., 1986)] and extended these studies into the chromatin from brain, because chromatin from neuronal nuclei is known to have several structural differences from other chromatins (Pearson et al., 1984). The data show that a number of metallic cations can cause marked changes in the degree of compaction of chromatin, resulting in its precipitation. In general, the ability of the cations to induce chromatin compaction and precipitation is related to their ionic radius and charge with aluminum, by virtue of its high charge density and low ionic radius being the most effective, particularly in brain. Since "heavy metal" cations can readily penetrate the cell nucleus (Bryan, 1980), it is conceivable that some of their toxic effects are due to disruption of the normal mechanisms of gene expression.

These observations, which demonstrate that aluminum has the highest affinity for chromatin of all the ions we tested, are important in relation to the known involvement of this cation

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