Preferential protection of the minor groove of non-operator DNA by lac repressor against methylation by dimethyl sulphate

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ABSTRACT

The binding of lactose repressor to non-operator DNA was studied by the modification of several DNA's, including glycosylated DNA, with dimethyl sulphate, which affects the minor and major grooves of DNA and single stranded DNA regions. The non-specific binding of the repressor to DNA protected the minor groove but apparently not the major groove of the DNA double helix against methylation and did not increase the content of single stranded DNA regions. This suggests that the repressor on binding to non-operator DNA makes contacts mainly in the minor groove of DNA and does not uncoil the DNA double helix. This is different from the interaction of the repressor with lactose operator DNA which occurs, as shown by Gilbert et al. (1), along both the major and the minor groove.

I. INTRODUCTION

Recently Gilbert et al. (1) have shown that lac repressor bound to lactose operator DNA specifically protects against methylation with dimethyl sulphate (Me₂SO₄), four guanines in the major groove and three adenines in the minor groove of the operator DNA double helix. At the same time the repressor enhances the methylation of two guanines and one adenine of the operator. Therefore, the lac repressor appears to interact with the operator DNA in both the minor and the major groove.

In an E. coli cell one lac repressor molecule is bound to the lac operator DNA with very high affinity (dissociation constant, K = 10⁻²³ M) (2). About 10 other repressor molecules in the cell (3) are not free but seem to be bound much less strongly to non-operator regions of DNA and this binding has been suggested to have functional significance (4). The repressor binds rather well to many different DNA's and synthetic polynucleotides with more preference for poly [d(A\+T)] (K = 10⁻⁸ M) than for poly [d(G\+C)] (K = 10⁻⁵ M) (5-6).

Here we report the results of methylation with dimethyl sulphate of the complex of the lac repressor with non-operator DNA. The results show that the repressor protects the minor but apparently not the major groove.
of DNA against methylation and that repressor binding to non-operator DNA does not cause uncoiling of the double helix.

II. MATERIALS AND METHODS

[3H]-dimethyl sulphate (140 mCi/m mole) was purchased from Amersham (England). The lac repressor was prepared as described (7). DNA from calf thymus and from Tetrahymena pyriformis GL was isolated as described (9). Glucosylated DNA of T4 phage and non-glucosylated DNA of T4 phage arg am8 Bgt am10 deficient in glucosylation (8) were prepared in the same way. To denature calf thymus DNA it was heated in 0.01 M sodium cacodylate, pH 7.4 at 100°C for 5 min and then quickly cooled in ice.

The methylation with dimethyl sulphate was carried out in 5 mM MgCl₂, 0.1 mM EDTA, 0.3 mM dithiothreitol, 100 mM KCl and 25 mM sodium cacodylate, pH 7.4 (binding buffer). 0.3 A₂₆₀ units of DNA (about 15 µg) were mixed with 100 µg of lac repressor in 0.25 ml of binding buffer. 5'-riboguanylic acid (GMP) (1.2 A₂₆₀ units) was introduced in the incubation mixture as an internal standard for estimation of the level of DNA methylation (9). After incubation for 15 min at 20°C the mixture was cooled to 4°C and [3H]-dimethyl sulphate was added to a concentration of 5 mM in 5 µl of dimethylformamide. After methylation for 18 hr at 4°C the concentration of EDTA in the incubation mixture was adjusted to 5 mM and DNA and protein were precipitated with 2 volumes of ethanol. The guanine of GMP was isolated from the supernatant by absorption of the products of acidic hydrolysis (1 N HCl, 100°C, 1 hr) on a column of Dowex 50 x 8. The incorporation of [3H]-methyl groups in GMP was measured as described (9). The precipitate was dissolved in 0.1 ml of 5 M urea, 2 M KCl, 0.1% cetavlon and 0.02% pronase by incubation at 4°C for an hour. Then the mixture was diluted 5-fold with water and the precipitate of the cetavlon salt of DNA was collected by centrifugation, washed twice with 1 M sodium acetate in 75% ethanol, twice with 95% ethanol and vacuum dried. The hydrolysis of DNA with HClO₄ and separation of methylated bases was carried out as described (10). Control samples contained either no protein at all or contained bovine serum albumin (at the same concentration as repressor).

To prepare an insoluble complex of calf thymus DNA with denatured bovine serum albumin, a mixture of 100 µg of DNA and 800 µg albumin in 0.1 ml of 0.01 M NaCl was heated at 55°C for 1 hr. Then 1 µl of 0.5 M sodium cacodylate buffer, pH 5.25, was added. The precipitate was centrifuged and 5 µl of 0.5 M sodium cacodylate buffer, pH 7.0, was added; the methylation was carried out as before.
III. RESULTS AND DISCUSSION

As established by Lawley and Brooks (11, see also review 12) dimethyl sulphate methylates DNA mainly at the N7 atom of guanine exposed in the major groove of the DNA double helix, at the N3 atom of adenine exposed in the minor groove, and at the N1 of adenine which is accessible to methylation in single stranded DNA regions. These methylations have recently been used to study the blocking of the minor and the major grooves by ligands and to measure single stranded regions in DNA complexed with different ligands, e.g., histones, protamines, polylysine, antibiotics, etc. (10,13, 14).

Table 1 shows the methylation of different DNA's in the presence or absence of lac repressor. In all cases the presence of repressor on the DNA (one tetrameric repressor per about 30 base pairs) increased the ratio of 7-methylguanine$(^7$G) to 3-methyladenine$(^3$A) by about 15% over the $^7$G/$^3$A ratio obtained after methylation of repressor-free DNA.

Table 1. The methylation of different DNA's in a complex with the repressor

<table>
<thead>
<tr>
<th>DNA</th>
<th>GC content</th>
<th>Methylation increase by repressor (%)</th>
<th>$^7$G/$^3$A</th>
<th>Protection of the minor groove by repressor (%)</th>
<th>$^7$G/$^3$A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tetrahymena</td>
<td>30</td>
<td>4.9±0.1</td>
<td>5.0±0.1</td>
<td>16</td>
<td>35±10</td>
</tr>
<tr>
<td>Calf thymus (native)</td>
<td>43</td>
<td>6.0±0.1</td>
<td>7.1±0.2</td>
<td>15</td>
<td>60±10</td>
</tr>
<tr>
<td>Calf thymus (denatured)</td>
<td>-</td>
<td>6.1±0.3</td>
<td>-</td>
<td>2.0±0.2</td>
<td>-</td>
</tr>
<tr>
<td>Tλ phage (glucosylated)</td>
<td>35</td>
<td>3.9±0.2</td>
<td>3.2±0.3</td>
<td>18</td>
<td>3.2±0.3</td>
</tr>
<tr>
<td>Tλ phage (non-glucosylated)</td>
<td>35</td>
<td>4.1±0.1</td>
<td>4.6±0.1</td>
<td>11</td>
<td>4.6±0.1</td>
</tr>
</tbody>
</table>

The increase of DNA methylation by repressor was calculated on the basis of GMP methylation which was found to be equal both in the presence of the DNA-repressor complex or repressor-free DNA. GMP was added to the incubation mixture as an internal standard (9). Under the conditions of these experiments the incorporation of 3H-CH$_3$ groups in free DNA was (mmol CH$_3$/mol P-DNA): GMP-10; DNA of Tetrahymena-2.0; DNA of calf thymus-2.5; glucosylated DNA of Tλ phage-1.5; non-glucosylated DNA of Tλ phage-2.0.

**Protection (P) of the minor groove against the methylation was calculated according to formula:**

$$P = \frac{R^+\text{Rep} - R^-\text{Rep}}{R^-\text{Rep}} \times 100\%,$$

where $R^+\text{Rep}$ and $R^-\text{Rep}$ are $^7$G/$^3$A ratios of DNA methylated in the presence and in the absence of repressor, respectively. In these calculations the major groove of DNA was not considered to be protected.
Under trace labeling conditions of methylation used in these experiments approximately one methyl group is incorporated per 500 nucleotides. In these conditions the decrease of methylation of DNA grooves in the presence of ligands bound to DNA is proportional to the relative amount of DNA which is shielded against methylation by ligands at any one time. The methylation decrease does not depend upon the length of reaction and $\text{Me}_2\text{SO}_4$ concentration. These conclusions follow from kinetic experiments on methylation of soluble chromatin in which protection of the major groove of DNA by histones (by 14%) was not changed during modification from 4 to 48 hr (9). Also, in reversible complexes of DNA with actinomycin D (14) or ethidium bromide (9) both ligands substantially protect the minor DNA groove against methylation by 30% and 78%, respectively. Under the present conditions of the reaction the repressor retained most of its activity. The increase in the $m^7\text{G}/m^3\text{A}$ ratio upon repressor binding to DNA suggests either the greater protection of the minor groove relative to the major groove against methylation or the greater stimulation of methylation of the major groove in comparison with that of the minor groove of DNA by lac repressor. On the basis of data described below the first suggestion seems to be much more reasonable despite the fact that the binding of the repressor enhances the total level of DNA methylation. The presence of repressor bound to DNA increased the level of DNA modification by 30-40%. This increase could be caused mainly by two different factors: either by a change of the B configuration of DNA or by an increase of the $\text{Me}_2\text{SO}_4$ concentration near the DNA (9).

Non-specific binding of repressor to poly d(A-T) appears to induce conformational changes in DNA as was suggested by Maurizot, Charlier and Hélène (15). But these conformational changes seem unlikely to produce enhancement of DNA methylation since conversion of DNA from the B form to other known forms, i.e. single stranded DNA or the C or A configuration, do not appear to increase formation of $m^7\text{G}$ or $m^3\text{A}$ upon DNA methylation (9).

On the other hand, the enhancement of DNA methylation is most likely explained by the absorption of $\text{Me}_2\text{SO}_4$ molecules by hydrophobic regions of repressor bound to DNA. This could result in a local increase in dimethyl sulphate concentration near to DNA, thereby enhancing DNA methylation. Table 2 illustrates this point. The presence of bovine serum albumin in solution with DNA influenced neither the $m^7\text{G}/m^3\text{A}$ ratio nor the level of DNA methylation. Denatured albumin, which bound to DNA in a precipitated complex on the other hand increased the DNA methylation by 10%, but did not change the $m^7\text{G}/m^3\text{A}$ ratio and therefore did not seem to affect preferentially.
Table 2. Methylation of calf thymus DNA in solution in the presence of native bovine serum albumin or in insoluble complex with denatured albumin

<table>
<thead>
<tr>
<th>Albumin</th>
<th>Methylation increase by albumin (%)</th>
<th>m^7G/m^3A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native albumin in solution</td>
<td>0</td>
<td>6.0±0.1</td>
</tr>
<tr>
<td>Denatured albumin bound to</td>
<td>10</td>
<td>6.0±0.1</td>
</tr>
<tr>
<td>DNA in insoluble complex</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Arrangement of some molecules in the DNA grooves should obviously cause the shielding of the grooves and respective reduction of m^7G or m^3A formation within them. For complexes which form a precipitate or any other kind of second macro- or microphase, methylation of different regions of DNA would depend on distribution of MeSO₄ between two phases and the rate of MeSO₄ diffusion to the DNA. The diffusion of MeSO₄ molecules to adjacent minor and major grooves of the DNA should not, however, be different due to a rather short distance between them. Therefore one can expect that the m^7G/m^3A ratio of methylated bases formed within adjacent grooves should only depend upon their relative shielding but not upon diffusion or the local concentration of MeSO₄ molecules. For this reason measuring of the m^7G/m^3A ratio would permit a more accurate assessment of the groove shielding in comparison with measuring the absolute amount of methylated guanine and adenine formed. For example, in comparative kinetic experiments on methylation of soluble chromatin and free DNA with MeSO₄, no increase was found in methylation of chromatin over free DNA. Histones protected only the major but not the minor groove of DNA against the methylation by 14% whether the calculations were based on the measuring level of methylated bases formed or on the decrease in the m^7G/m^3A ratio. The similar protection by 17% of the major groove of DNA by histones was found in the case of precipitated reconstituted nucleohistone when the calculations were based on the change in the m^7G/m^3A ratio. However in this case DNA in a precipitate of reconstituted nucleohistone was methylated by 30% higher than DNA in soluble nucleohistone, apparently as a result of concentrating MeSO₄ in the solid phase. Similar enhancement of DNA methylation has been found in the case of other precipitating agents (9) which shield the major groove (protamine, polyarginine), the minor groove (cetyltrimethylammonium ions) or neither DNA groove (polylysine).
Taken together these data suggest that enhancement of DNA methylation by repressor is due to absorbing and concentrating Me$_2$SO$_4$ molecules in the complex. Therefore the increase in the $m^7G/m^3A$ ratio found for methylated DNA-repressor complex in comparison with repressor-free DNA appears to be caused by preferential protection of the minor groove relative to the major DNA groove by repressor. This increase in the $m^7G/m^3A$ ratio is difficult to explain by conformational changes in DNA induced by repressor, as transition DNA from the B configuration to single stranded form or the C configuration could produce no changes or only reduction in this ratio, respectively (9) and conversion from the B to the A configuration by repressor has not been experimentally supported (15).

Since the minor groove seems to be protected preferentially by repressor against the methylation it is important to assess the role of the major groove in the interaction of DNA with repressor.

The increase of DNA methylation in the presence of protein could be taken as a rough measure of the amount of protein bound. The repressor enhanced methylation to the same degree (by 30%) of both glucosylated and non-glucosylated DNA of T4 phage and hence seems to be equally bound to both DNA's. Also there is little difference in levels of shielding of the minor groove against methylation by the repressor between glucosylated (11%) and non-glucosylated (18%) phage DNA's, both of which also lie in the same range as this shielding of DNA of Tetrahymena or calf thymus (about 15%) (Table 1).

According to the data of Table 1 glucosyl residues bound to all 5-hydroxymethyl cytosines in the major groove of T4 phage DNA protect the major groove of DNA by 28% against the interaction with a low molecular weight substance such as dimethyl sulphate. But the presence of glucosyl groups, that screen the major DNA groove from the methylation by 28% and that should screen it even more from the interaction with a much more bulky repressor molecule, has essentially no influence on the protection of the minor groove by the repressor and perhaps on the binding of the repressor with DNA. Thus it seems that the repressor is not bound in the major groove of DNA. This finding agrees both with the results of Lin and Riggs (16), who found that glucosylated T4 DNA binds well to lac repressor, and with the recent observation by Richmond and Steitz (17) that in the binding of lac repressor to poly [d(A-U-HgX)] (X-mercaptans) the repressor does not detect blocking HgX groups in the major groove. Taken together with these data the protection of the minor groove against the methylation by the repressor suggests that the repressor makes some contacts with non-operator.
DNA (preferentially with A-T rich regions) mainly within this groove, but not within the major DNA groove. However, the repressor makes contacts with lactose operator DNA within both DNA grooves (1). To explain this contradiction we propose a two-step model of repressor binding to DNA.

Initially lac repressor interacts with the ribose-phosphate backbone and with DNA bases preferentially within the minor DNA groove. When the proper DNA segment can be roughly recognized in such a way through the minor groove, some conformational changes could occur which allow the repressor to surround the DNA double helix and interact with both DNA grooves.

It is of interest to note that the mode of binding of the repressor to non-operator DNA is different from that of some other polypeptides, since histones and polyarginine protect the major groove, and polylysine seems to protect neither DNA groove against methylation (9).

Since N1 of adenine is methylated in single stranded DNA regions and N7 of guanine is modified equally well in single and double stranded DNA the ratio of mG/mA reflects the amount of single stranded DNA present (10). For native DNA's tested in these experiments the mG/mA ratios lay in the range 35-60, but for heat denatured calf thymus DNA this ratio decreased essentially to value of 2.0. The binding of the repressor to any DNA investigated here did not change this ratio. This suggests that the repressor does not cause noticeable uncoiling of non-operator DNA upon its binding. This is consistent with the finding that the repressor binds well to poly d(A-U-HgX) in which both chains are stabilized against denaturation by cross-linking (17).

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