Photoaddition of trimethylpsoralen as a probe for the intracellular organization of *Escherichia coli* DNA

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ABSTRACT

It has been previously demonstrated that photoreaction with 4,5',8-trimethylpsoralen (trioxsalen) can be used as a probe for the in vivo structure of eucaryotic chromatin. We have used this probe to analyze the organization of intracellular *Escherichia coli* DNA. In contrast to eucaryotic DNA, bacterial DNA within the intact cell is not protected from saturating doses of trioxsalen photoaddition. The final level of covalently bound trioxsalen upon saturation is identical to that found with purified DNA. In addition, the distribution of interstrand DNA cross-links formed by low doses of trioxsalen photoadducts does not exhibit the repeating pattern that has been observed with eucaryotic nucleosomes.

INTRODUCTION

Gentle lysis procedures have been used to isolate bacterial chromosomes in a folded and supercoiled form often called a "nucleoid" (1,2). By directly lysing *E. coli* cells onto electron microscope grids, Griffith has observed a chromosome structure that somewhat resembles the regular beaded structure of eucaryotic chromatin (3). Although bacteria lack the histones of eucaryotes, several low-molecular weight basic DNA-binding proteins have been identified in *E. coli* (4-6). Some of these proteins have been shown to be associated with the bacterial nucleoid (7,8). Recently, it has been reported that one of these proteins, the *E. coli* DNA-binding protein HU, has a beaded appearance in glutaraldehyde-fixed preparations visualized by electron microscopy, and resembles in many respects the in vivo nucleoprotein complex ("minichromosomes") of SV40 DNA and the four cellular core histones (9-12).

In order to determine whether there is a regular nucleosome-like organization of bacterial DNA in situ, we have analyzed the structure of *E. coli* "chromatin" by the trioxsalen photoreaction of DNA in the intact bacterial cell. The approach we have taken is to photoreact the cells with derivatives of furocoumarins (psoralens), a class of compounds which readily penetrate

intact cells or virus particles, intercalate into the DNA, and in the presence of long-wavelength UV light (365 nm), covalently add to the pyrimidines of the DNA (13-16). If two pyrimidines are adjacent and on opposite strands, the two DNA strands can be covalently cross-linked (17-19).

It has been demonstrated that one of these derivatives, 4,5',8-trimethylpsoralen (trioxsalen), is a particularly valuable probe for the structure of eucaryotic chromatin (20). The DNA within a nucleosome structure is preferentially protected from photochemical addition in cellular chromatin (21) and in the intracellular form of the SV40 minichromosome (22). Eucaryotic chromatin, when analyzed with this technique, exhibits two properties that distinguish it from naked DNA. The level of photoaddition at saturation following multiple additions of drug and prolonged irradiation is approximately one trioxsalen molecule per 40 base pairs, in contrast to the level of one in 4-8 base pairs for purified DNA. In addition, the pattern of cross linking, as determined by electron microscopic analysis of the loops that result upon denaturation of the DNA, has a regular repeat unit of 160-200 base pairs (21-23).

We have determined these two parameters for E. coli DNA in intact bacterial cells and found that the properties of bacterial DNA are much more like that of naked DNA than that of eucaryotic chromatin. This result may illustrate a fundamental difference between histone-complexed DNA and DNA that is neutralized and condensed in the presence of procaryotic low-molecular-weight basic DNA-binding proteins.

MATERIALS AND METHODS

Bacteria

For the saturation experiments Escherichia coli strain QD (obtained from H. Echols) was used. In the electron microscopic analysis of the distribution of cross-links, strain K12DG75 (obtained from J. Clark) was employed.

Photoaddition of trioxsalen: saturation experiments

Bacterial cultures were grown to a density of $1-2 \times 10^9$ cells/ml in minimal medium MM9 (24) supplemented with 0.3% glycerol, 0.3% Difco Bacto Casamino acids and 5 μ g/ml thiamine hydrochloride. The cells were then concentrated 10-20 fold by centrifugation and resuspended in unsupplemented M9 salts. Irradiations were carried out at 4°C in closed tissue culture dishes resting on two General Electric F20T12-BLB fluorescent tubes at an incident intensity of approximately 3 mW/cm^2 . ^3H -labelled trioxsalen was added in sequential doses of 1 μ g/ml each from a 0.5 mg/ml ethanol stock solution. The cells

were washed by centrifugation after every ten additions so that the ethanol concentration never exceeded 2.5%. Following each addition of trioxsalen, the cells were irradiated for 5 min. After every tenth addition the irradiation time was increased to 30 min to insure completion of the photochemical reaction.

Photoaddition of trioxsalen: electron microscopic analysis of crosslinks

Bacterial cultures were grown to a density of 5×10^8 /ml in supplemented tryptone broth (10 g tryptone and 5 g NaCl/l, 20 μ g/ml thymine, 10 μ g/ml thiamine hydrochloride), centrifuged and resuspended in 0.01 M Tris-hydrochloride pH 8.2, 0.01M NaN_3 , 0.1M NaCl and 20% sucrose at a density of 2×10^9 cells/ml. Cells were irradiated in the presence of trioxsalen as described in Figure 3 with constant mixing in the "rocking device" described previously (20).

Isolation and purification of bacterial DNA

Following irradiation the cells were resuspended in L buffer (0.05M Tris-hydrochloride pH 8.1, 0.001M NaN_3 and 25% sucrose) at approximately 10^{10} cells/ml, digested with lysozyme at 1 mg/ml for 5 min on ice, adjusted to 0.05M EDTA and digested 15 min further on ice. The samples were then treated with 1.5% sarkosyl (Geigy) and 1 mg/ml pronase (Calbiochem, grade B, predigested for 30 min at 37°C) at 50°C for 3-5 hr. The DNA was then banded by density equilibrium gradient centrifugation in Cs_2SO_4 (2.75g of Cs_2SO_4 added to 4.5 ml of DNA solution and centrifuged at 35,000 rpm for 48 h in a Beckman SW50.1 rotor at 17°C). The viscous DNA band was collected, dialyzed against TE buffer (0.01 M Tris-hydrochloride pH 8.1, 0.001 M EDTA), adjusted to 1M NaCl, extracted three times with chloroform-isoamyl alcohol (24:1) to remove free ^3H -trioxsalen, and dialyzed against TE buffer.

Quantitation of bound trioxsalen

Trioxsalen was obtained from the Paul B. Elder Co. (Bryan, Ohio) and tritiated in this laboratory by S. Isaacs (25). Stock solutions of approximately 0.9 mg/ml were made in ethanol. Specific activities were determined by counting in 1 ml of water and 10 ml of Omnifluor-Triton scintillation fluid (2 liters of toluene, 1 liter of Triton X-100, and 12 g of Omnifluor (New England Nuclear) on a Beckman LS-230 scintillation counter) and measuring the absorbance at 249 nm. The extinction coefficient in 100% ethanol at this wavelength is $31,000\text{M}^{-1}\text{cm}^{-1}$ (J.E. Hyde, personal communication).

DNA concentrations were determined using an extinction coefficient of $6,000\text{M}^{-1}$ phosphorous (26) (50 μ g/ml per optical density unit at 257 nm in

a cuvette with a path length of 1 cm).

The number of base pairs per bound trioxsalen molecule was calculated by determining the specific activity of the purified DNA following irradiation.

Electron microscopy

The purified DNA samples were denatured in 0.5 M glyoxal-70% formamide-0.01 M sodium phosphate (pH 7.0)-1 mM EDTA for 60 min at 40°C (27) and spread in formamide according to the method of Davis et al. (28) by using a modification of the Kleinschmidt procedure (29). Grids were rotary shadowed with 80% Pt-20% Pd or with tungsten, and microscopy was carried out on a Philips 201 electron microscope.

Histograms of loop length were generated by projecting 35-mm negatives and measuring with an electronic planimeter (Numonics Corp.) interfaced to a paper-tape punch. A PDP 8/E computer was used to calculate and compile the data into the form of histograms in base-pair units.

RESULTS

Comparison of trioxsalen photoaddition in intact and permeabilized cells

In order to determine whether the DNA of bacterial cells was protected from trioxsalen photoaddition to the same extent as eucaryotic chromatin, *E. coli* cells were treated with ^3H -labelled trioxsalen and irradiated with long-wavelength UV light (340-380 nm). Following DNA purification and extraction of unbound trioxsalen, the amount of covalently bound drug was determined. Because the solubility of trioxsalen in aqueous solutions is limited (0.6 $\mu\text{g/ml}$), it is necessary to add successive additions of a saturating dose (1 $\mu\text{g/ml}$) at 5 or 10 minute intervals of irradiation to achieve the maximum level of photoaddition. Since the cell wall and cell membrane might be expected to provide a significant kinetic barrier to trioxsalen entry, the extent of photoaddition after a single drug addition was first compared in three systems: intact cells, toluene-treated cells, and lysozyme-digested cells. The results are shown in Table 1. Since there was little additional drug bound by the spheroplast DNA, and in fact less drug bound by the DNA of the toluene-permeabilized cells, intact cells were employed for all subsequent experiments. The irradiations were carried out in M9 salts in order to further minimize the effects of prolonged incubation on cellular structure.

Saturation experiments of ^3H -trioxsalen photoaddition to *E. coli* DNA

The kinetics of ^3H -trioxsalen photoreaction with bacterial DNA *in situ* was compared to that with purified *E. coli* DNA. The results of these ex-

TABLE I. Comparison of Trioxsalen Photoaddition in Intact and Permeabilized Cells*

	Irradiation Time	³ H-cpm/ 100 μ l	trioxsalen μ g/ml	DNA μ g/ml	Base pairs/ trioxsalen
Intact	5'	4180	0.0740	58.5	274
	30'	9353	0.1655	78.5	164
Toluene	5'	2159	0.0382	59.2	536
	30'	3833	0.0678	68.5	350
Lysozyme	5'	5269	0.0933	46.0	171
	30'	4927	0.0872	37.5	149

* *E. coli* cells, concentrated ten-fold in L buffer, were divided into three aliquots: (1) intact cells, (2) toluene-treated cells, to which was added toluene to a final concentration of 1% and (3) lysozyme-treated cells which were digested with lysozyme in the presence of EDTA as described for DNA purification in the Materials and Methods section. A single addition of 2 μ g/ml of ³H-trioxsalen (565,000 cpm/ μ g) was then made and the samples were irradiated for the times indicated. The cells were then lysed, the DNA purified and the amount of bound trioxsalen determined as described in Materials and Methods. The number of base pairs/bound trioxsalen has been calculated assuming a molecular weight ratio of DNA base pair/trioxsalen of 660/228 or 2.89.

periments are compiled in Tables 2 and 3 and summarized graphically in Figure 1 in base pairs per bound trioxsalen as a function of the number of additions. It can be seen that at low trioxsalen doses, the number of base pairs per bound trioxsalen molecule is considerably greater for the DNA treated in whole cells (Table 2) than for purified DNA (Table 3). However, after further additions of drug, the saturation level of bound trioxsalen is similar to that of naked DNA (approximately 6-10 base pairs per trioxsalen in these experiments). Thus, in contrast to eucaryotic DNA, which exhibits a plateau at a saturation level 10% that of purified DNA (21,22), the apparent protection of *E. coli* DNA from photoaddition at early time points is only a function of a kinetic barrier. The final level of photoadducts at saturation is similar to that of purified DNA in solution.

TABLE 2. Saturation Experiments of ^3H -trioxsalen Photoaddition to Intact *E. coli* Cells*

Experiment	Number of additions	cpm*, †, ‡	trioxsalen $\mu\text{g/ml}$	DNA $\mu\text{g/ml}$	Base pairs/trioxsalen
I*	24	1,670	0.1182	16.9	49.5
	36	2,949	0.2088	23.0	38.1
	48	4,108	0.2908	18.4	21.9
II†	5	537	0.0443	63.3	494.4
	10	1,285	0.1060	62.5	204.0
	20	3,469	0.2861	79.0	95.5
	30	7,608	0.6275	91.0	50.2
	40	10,973	0.9050	85.8	32.8
	50	28,132	2.320	150.0	22.6
III‡	10	2,003	0.0436	16.7	132.5
	20	3,361	0.0732	12.4	58.6
	40	12,882	0.2807	12.5	15.4
	60	6,459	0.1407	4.7	11.6
IV‡	10	3,108	0.0677	16.4	83.8
	20	10,903	0.2375	20.4	29.7
	40	41,798	0.9106	43.7	16.6
	60	45,619	0.9939	25.9	9.0
	80	143,313	3.122	59.3	6.6

* Cells were grown and irradiated in the presence of ^3H -trioxsalen as described in detail in the Materials and Methods section. The number of base pairs per trioxsalen was calculated as in Table 1. The actual volumes of DNA counted and the specific activities of the drug employed in each experiment were as follows: *25 μl counted, ^3H -trioxsalen 565,000 cpm/ μg ; †25 μl counted, ^3H -trioxsalen 485,000 cpm/ μg ; ‡100 μl counted, ^3H -trioxsalen 459,000 cpm/ μg .

Photoaddition of trioxsalen at presaturation levels: electron microscopic analysis of cross-links

Another approach to the analysis of the intracellular structure of bac-

TABLE 3. Saturation Experiments of ^3H -trioxsalen Photoaddition to Purified *E. coli* DNA*

Experiment	Number of additions	^3H -cpm /100 μl	trioxsalen $\mu\text{g/ml}$	DNA $\mu\text{g/ml}$	Base pairs/trioxsalen
III	2	4,947	0.1078	21.4	68.7
	5	11,216	0.2444	20.2	28.6
	10	18,594	0.4051	20.4	17.4
	20	19,933	0.4343	20.0	15.9
IV	2	6,929	0.1510	37.5	85.9
	5	16,517	0.3598	35.6	34.2
	10	31,531	0.6869	35.6	17.9
	20	45,780	0.9974	31.3	10.9
	40	49,087	1.0694	25.8	8.3

* *E. coli* DNA was purified as described in Materials and Methods and irradiated in M9 salts and repurified in parallel with the intact cells of the same experiment number as that in Table 2. The DNA concentration during the irradiation was 50 $\mu\text{g/ml}$. The specific activity of the ^3H -trioxsalen in both experiments was 459,000 cpm/ μg . The number of base pairs per trioxsalen was calculated as in Table 1.

terial DNA is to determine the distribution of cross-links introduced into the DNA by covalently bound trioxsalen. If the apparent protection from photoaddition at early irradiation times is due to a regular repeating structure, the histogram of the distance between cross-links will appear as a nonrandom pattern with a monomer peak at the position of the size of the protected unit. This repeating unit for eucaryotic chromatin is the nucleosome, and as described in the Introduction, histograms of the distance between cross-links generated by treating eucaryotic cells or intracellular SV40 nucleoprotein complexes with trioxsalen and long-wavelength UV light, have a monomer peak of 160–200 base pairs and occasionally a dimer peak of 350–400 base pairs at low trioxsalen doses.

The pattern of cross-links generated by the photoaddition of trioxsalen

Figure 1. Kinetics of ^3H -trioxsalen photoaddition to *E. coli* DNA. The data in this graph is a compilation of the values presented in Tables 2 and 3. The individual experiments are represented by the following symbols: Intact cell irradiation (Table 2): (■), I; (X), II; (●), III; (▲), IV. These points are connected by a solid line. Purified DNA irradiation (Table 3): (○), III; (Δ), IV. These points are connected by a dashed line.

at presaturation levels was determined electron microscopically as described in the Materials and Methods section. Under denaturing conditions, the purified DNA forms a pattern of loops, the arms of which represent the two denatured strands between two cross-links (Figure 2). Figure 3 illustrates two histograms of the distribution of these loop sizes. In Figure 3A two additions of drug and total irradiation time of 175 min were employed, whereas in Figure 3B, the cells were treated with a single dose of trioxsalen for a shorter irradiation time. The extent of photoaddition could not be determined as accurately in these experiments as for those in Tables 1-3 because unlabelled trioxsalen was used. However, the DNA in Figure 3A was 51% and that in 3B was 15% double-stranded in the electron micrograph under denaturation conditions. These levels are comparable to the early levels of photoaddition in the saturation experiments previously described.

As can be seen from the data presented in Figure 3, the number of base pairs at the peak of the histogram (i.e., the most frequently represented

Figure 2. Electron micrograph of cross-linked *E. coli* DNA. The DNA from the experiment described in Figure 3B was denatured and spread as described in the Materials and Methods section. The length bar represents 1000 bases and was determined by measurement of circular fd bacteriophage DNA marker molecules (length=6389 bases) such as that seen near the center of the micrograph.

size class) decreases at increased drug dose. This is in contrast to the situation in eucaryotic cells where an increased level of photoaddition results in a change in the proportion of monomer and dimer peaks, but does not produce a shift in the peak position (20,22). It should also be noted that the peaks in these experiments are broad and very similar to those observed when purified DNA is cross-linked *in vitro* at different levels of photoaddition (22).

DISCUSSION

It has been shown that *E. coli* DNA is not protected *in vivo* from photochemical addition of trimethylpsoralen. In saturation experiments with multiple additions of drugs, the final quantity of ^3H -trioxsalen bound to the bacterial DNA is indistinguishable from the levels obtained with puri-

Figure 3. Histograms of the loop lengths of *E. coli* DNA cross-linked in the intact cell. *E. coli* DNA was prepared and spread for electron microscopy and the resulting denatured loops were measured and analyzed as described in Materials and Methods. In experiment A the cells were irradiated for 175 min; 5 $\mu\text{g/ml}$ trioxsalen was added at time 0 and again at 60 min; 894 loops were measured. In experiment B, the cells were treated with a single dose of 5 $\mu\text{g/ml}$ trioxsalen and then irradiated for 60 min; 1585 loops were measured.

fied DNA. In contrast, eucaryotic DNA is protected from photochemical addition and a plateau level of DNA base pairs per adduct is obtained that is ten fold higher than that observed with naked DNA.

The apparent kinetic difference in photoaddition between the "*in vivo*" and *in vitro* experiments (Tables 2 and 3) may be due to factors other than structural differences between purified and intracellular *E. coli* DNA. These include (1) permeability barriers, (2) absorbance or scattering of light by the bacterial suspension, and (3) uptake of the drug by cellular RNA. That this difference is due to permeability barriers appears unlikely in light of the results of Table 1. It was found that digestion of the cell wall had only a very small effect on ^3H -trioxsalen photoaddition. Permeabilization of the cells by 1% toluene, a technique that has been exploited in order to enable cells to take up deoxynucleotide triphosphates (30), actually decreased the extent of photoaddition.

The results of analyzing the distribution of trioxsalen cross-links are consistent with a random distribution in which the average distance between

the cross-links decreases with an increase in the amount of trioxsalen bound. Thus, in contrast once again to eucaryotic chromatin, the properties of intracellular E. coli DNA closely resemble those of purified DNA in this system, in spite of the apparent "nucleosome-like" structure observed by electron microscopy under gentle lysis conditions (3).

These conclusions do not necessarily indicate that there are no proteins bound to E. coli DNA inside the cell. They simply show that such proteins, if closely associated with the DNA, do not confer the type of protection from trioxsalen photoaddition that has been seen with DNA in a nucleosome form. If a transient protection is the mechanism for the decreased level of photoadducts at low doses, the results in Figure 3 imply that such protection does not provide a repeating array of regular subunits, in contrast to the results obtained with the in vitro reconstitution system of Rouviere-Yaniv et al. (9). It would be interesting to test such a reconstituted complex of DNA and HU protein with trioxsalen photoaddition.

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