Photochemical Cross-Linking of *lac* Repressor to Nonoperator 5-Bromouracil-Substituted DNA†

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ABSTRACT: Ultraviolet irradiation of *lac* repressor bound to 5-bromouracil-substituted nonoperator DNA leads to the formation of cross-links between the protein and the nucleic acid. The cross-links are formed between the DNA and the 1–51 N-terminal peptide of the repressor, the "headpiece". The tetrameric core (4 × 60–360 amino acids) was never found to be cross-linked to the DNA. With isolated headpieces, which are able to bind DNA, no cross-link was detected. These results are discussed considering the fundamental role of the core in keeping the headpieces in an adequate geometry for the DNA–repressor interaction. It has been possible to cross-link two DNA molecules to one repressor molecule, thus showing the existence of at least two binding sites for nonoperator DNA on the repressor. The attached peptides were analyzed after extensive proteolytic cleavage, and the most abundant peptide found was peptide 23–33. Histidine-29 seems to be the photo-cross-linked amino acid. Analysis of the results required a computation method discussed in the Appendix.

The nonspecific binding of *lac* repressor to nonoperator DNA plays a role in operator–repressor recognition (Richter & Eigen, 1974; von Hippel et al., 1974, 1975; Berg & Blomberg, 1977; Berg et al., 1981; Winter & von Hippel, 1981; Winter et al., 1981) and may serve as a model for specific binding studies. In the case of the specific operator–repressor interaction, several contact points on the operator have been identified [see Barkley & Bourgeois (1978)], especially by the study of methylation patterns (Ogata & Gilbert, 1978, 1979). No data are available, however, on the contact points on the repressor, except that the headpieces (1–51 or 1–59 N-terminal peptides) are considered to be the major interacting domains. Genetic studies have allowed identification of a set of amino acids of critical importance for the interaction (Miller et al., 1979; Miller & Schmeissner, 1979; Miller, 1979); this does not prove, however, that they are the contacts points, since they may also play a conformational role in maintaining the interacting structure.

Some years ago, Lin & Riggs (1974) reported a photochemical cross-linking of the *lac* repressor to operator bearing DNA substituted with 5-bromodeoxyuridine, without any further inference concerning the contact points. More recently, Ogata & Gilbert (1977) using a 55 base pair fragment bearing the operator have determined the 5-BrU-substituted thymines which can photo-cross-link the repressor.

This paper describes an attempt to delimit the region of the repressor attached to the photo-cross-linked nonoperator DNA in order to obtain further information about the residues of the protein implicated in the interaction.

Materials and Methods

Wild-type repressor protein was purified from BMH 493 strain by the procedure of Rosenberg et al. (1977) modified in our laboratory by Culard & Maurizot (1981). The repressor was checked to be fully active for inducer and operator binding.

The short headpiece was prepared according to Ogata & Gilbert (1979). Its activity for nonoperator DNA was controlled by observing the increase of the circular dichroism (CD) signal of DNA (Schnarr et al., 1983).

5-Bromouracil-substituted DNA (BrU-DNA) was purified from *Escherichia coli* B/rT⁻ strain. M9 growth medium (Miller, 1972) was supplemented with 4 μg/mL 5-bromodeoxyuridine. The percentage of substitution was determined by analytical ultracentrifugation in CsCl gradients (Hackett & Hanawalt, 1966) and found to be 72%.

Complexes between BrU-DNA and the repressor or headpiece were prepared by mixing the two components in a high ionic strength buffer (0.2 M potassium phosphate, pH 7.25, and 10^{-4} M dithioerythritol) followed by extensive dialysis against a low ionic strength buffer (1 mM potassium phosphate, pH 7.25, and 0.1 mM dithioerythritol). Typical ratios of 1 repressor to 25 base pairs or 1 headpiece to 6 base pairs were used.

Irradiations were performed by using an Osram HbO 200-W mercury lamp with MTO J 310a and MTO H 325a filters to isolate an irradiation band ranging from 300 to 400 nm (see Figure 1). This procedure was used to limit the photolysis of the repressor (Charlier et al., 1977). As the BrU-DNA absorbs at wavelengths longer than normal DNA (Boyce & Setlow, 1963) due to bromine substitution, the efficiency of the photochemical process remains large enough in this irradiation range, as previously observed for bacteria and phage inactivation (Boyce & Setlow, 1963; Setlow & Boyce, 1963).

The amount of cross-linked repressor was assayed by the following procedure. The irradiated complex (samples of 2 mL, 0.1 mg/mL DNA, and 1.8 mg/mL repressor) was passed through a Sepharose 6B column (1.5 × 25 cm). When the column was equilibrated and eluted with a low ionic strength buffer (1 mM potassium phosphate–0.1 mM dithioerythritol), both cross-linked and non-cross-linked repressors were eluted with the DNA, in the excluded volume. At high ionic strength (0.2 M potassium phosphate, 0.5 M KCl, and 10^{-4} M dithioerythritol), the non-cross-linked repressor was delayed from the DNA–cross-linked repressor in a well-separated peak, allowing quantitative determination. The absorbances were measured at 260 and 280 nm. The fluorescence (Figure 1) due to tryptophan-containing peptides (i.e., the repressor and, more precisely, the core) was measued at 340 nm (excitation at 290 nm). For the detection of the headpieces, which contain only tyrosine, the fluorescence was measured at 310 nm (excitation at 275 nm).

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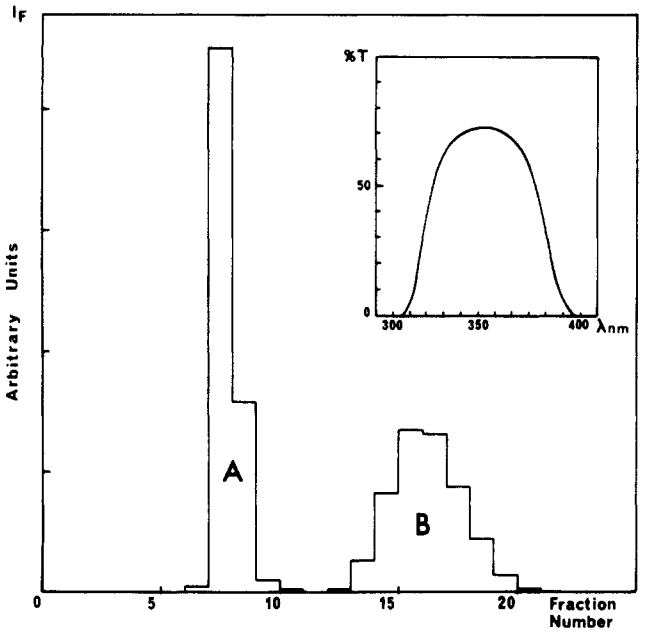

FIGURE 1: Elution pattern on Sepharose 6B at high ionic strength of repressor–BrU-DNA irradiated at low ionic strength. I_F is the fluorescence intensity at 340 nm (see Materials and Methods). Inset: Transmittance spectrum of the filter system (H 325a + J 310a filters).

Proteolysis (trypsin or chymotrypsin) of the repressor was carried out by two alternative methods. A limited digestion keeping the core and the headpieces intact was made in 1 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.25, and 30% glycerol (Geisler & Weber, 1977, 1978). Extensive digestion in 0.1 M ammonium bicarbonate at 25 °C (Platt et al., 1973) cleaved the headpieces to their tryptic or chymotryptic peptides but did not attack the core.

Electrophoreses were performed with a 7.5% polyacrylamide gel [30/0.8 acrylamide/bis(acrylamide)] in a buffer of 0.1 M sodium phosphate–1% sodium dodecyl sulfate. Refrigerated plates were run for 7 h at 80 mA.

Digestion of cross-linked DNA by DNase I (2.5% w/w) was carried out at 34 °C for 24 h in 10 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, and 0.1 mM dithioerythritol. At this low ionic strength, the adduct of repressor and oligonucleotides is not soluble. Insoluble material was centrifuged and extensively rinsed with 1 mM potassium phosphate and 0.5 mM ethylenediaminetatraacetic acid (EDTA) until no DNase activity was present. The "cross-linked complex" can be resolubilized by adding DNA (see Results).

The peptides cross-linked to the DNA were prepared as follows. After non-cross-linked repressors were removed by gel filtration at high ionic strength, the material was dialyzed against 0.1 M ammonium bicarbonate, pH 8. The first tryptic digestion was made by using 2% (w/w) trypsin for 2 h at 37 °C. The solution was then heated in boiling water for 3 min and rapidly cooled to denature the proteins and the DNA. Denatured enzyme and denatured core, which are precipitated, were discarded after centrifugation. The supernatant was digested a second time using 1% (w/w) trypsin (calculated on the basis of the headpieces) for 4 h at 37 °C and then for 16 h at 25 °C. The solution was adjusted to 0.2 M potassium phosphate, 0.5 M KCl, and 0.1 M NaOH and passed through a Sephadex G100 column equilibrated with 0.1 M triethylamine and 0.9 M KCl pH 11. This procedure is used to peel the DNA from all non-cross-linked peptides which could remain complexed to it. The DNA–peptide material, eluted in the excluded volume, was desalted on Sephadex G50 eluted with double-distilled water. Samples were then lyophilized.

After acid hydrolysis (5.6 N HCl for 36 h at 110 °C), the amino acid composition was determined by using a Biotronik LC 6000 E automatic amino acid analyzer according to standard procedures.

Amino acid analysis of BrU-DNA alone, treated and hydrolyzed under the same conditions, leads to the detection of numerous products, some of them being abundant. Some of these products eluted as expected for ammonium, glycine, or serine, for instance. It was thus necessary to use BrU-DNA alone as a blank. The standardization was made by using the area of the peak of glycine, since the contribution of the peptides appears to be negligible compared to that of DNA alone in this peak.

Five different analyses corresponding to five different preparations of complex and digestions gave exploitable results. Tryptophan, phenylalanine, and cysteine were absent, as presumed (the headpiece does not contain these residues). The amino acid composition did not correspond to only one unique tryptic peptide of the headpiece, and we therefore considered that it was a mixture of several peptides. The solution to this problem was found by decomposing the experimental mixture of amino acids on a base of peptides considered as independent (since neither of them is a linear combination of the others). The number of amino acids was reduced to 12 for experimental reasons. Trp, Phe, and Cys were absent. Pro was too insensitive to ninhydrin to give precise results, Tyr was susceptible to photolysis, and Gly was much too abundant in the blank. Gln and Asn were transformed to Glu and Asp during acid hydrolysis. The number of independent tryptic peptides was six, as shown in Table I. The computation method was adapted from that described by Magar (1968) for optical rotatory dispersion (ORD) analysis of proteins (see the Appendix).

Results

Cross-Linking of Repressor–BrU-DNA Complexes. The formation of complexes at low ionic strength was checked before irradiation by circular dichroism (CD) spectroscopy. The change in the CD spectrum of BrU-DNA is quite similar to that obtained with other natural or synthetic DNAs (Maurizot et al., 1974; Durand & Maurizot, 1980). After 20-min irradiation, the decrease of the repressor fluorescence due to tryptophan photooxidation (Charlier et al., 1977) is less than 10%. Under these conditions, 60% of the complexed repressor is covalently bound to DNA. For irradiations longer than 20 min, the percentage of bound repressor does not increase significantly, whereas the photolysis of the repressor itself increases. Irradiation of the same DNA–repressor mixture at high ionic strength (when no complexes are formed) does not lead to any cross-link (Figure 2) since, in this case, the proximity of DNA and repressor is diffusion controlled.

Core Is Never Cross-Linked to DNA. To determine the cross-linked domain of the protein, proteolytic digestions of the cross-linked complex were performed after elimination of the noncovalently bound repressor by passing through Sepharose 6B at high ionic strength. After proteolysis, the solution is passed through the column for a second time at high ionic strength, and the core is eluted in a volume equal to that for intact repressor. Extensive digestion with trypsin shows that after 4 h, more than 99% of the core is eluted separately from the DNA peak (no fluorescence characteristic of tryptophan remains in the DNA peak). Electrophoretic analysis of this core shows that it is identical with that obtained by tryptic digestion of free repressor under the same conditions. Nevertheless, we note that after 40-min digestion, a shortened core (4 × 60–340) appears, as mentioned by Files & Weber

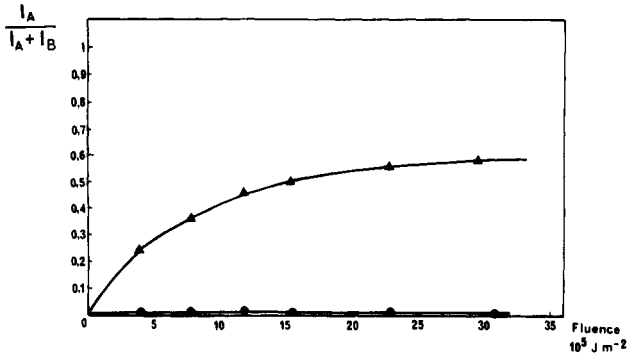

FIGURE 2: Kinetics of repressor–BrU-DNA cross-linking. $I_A/(I_A + I_B)$ is the relative area of the first peak compared to the sum of the areas of the two peaks from Figure 1. (▲) Irradiation of the complex at low ionic strength; (●) irradiation of the mixture at high ionic strength. A fluence of 10^5 J m^{-2} corresponds to an irradiation duration of 1 min under our experimental conditions.

(1976). At this time, 7% of the core remains attached to DNA. We cannot conclude unambiguously, therefore, that the headpiece is the only cross-linked domain, since the C-terminal part of the core might still be cross-linked to DNA.

To answer this question, chymotryptic digestions were carried out. Limited digestion of the cross-linked complex with chymotrypsin for 14 h leads to the release of more than 97% of the core (4 × 57–360) without any formation of the C-terminal-shortened core (Geisler & Weber, 1978). Since the only cleavage in this case is at leucine-56, we can conclude that the cross-linked domain of the repressor belongs to the headpiece.

Tentative Cross-Linking of the Headpiece. A ratio of one headpiece to six base pairs was chosen, and the formation of the complex was checked by circular dichroism measurements (Schnarr et al., 1983). After Sepharose 6B chromatography of the irradiated complex, the presence of the headpiece in the DNA peak was assayed by fluorescence measurements. Corrections for the screening effect, and for quenching by DNA binding (Schnarr et al., 1983), were made. Nevertheless, irradiations performed under the same conditions as for the repressor do not induce any cross-link. More than 93% of the input fluorescence was recovered in the peak of the headpiece, and only background fluorescence could be consistently detected in the DNA peak.

Number of Binding Sites on the Repressor. We prepared a batch of DNA cross-linked to repressor. The DNA was then digested as described under Materials and Methods to obtain repressors cross-linked to short polynucleotide chains. Following addition of free DNA and dialysis against low ionic strength buffer for 48 h at 4 °C, the sample (which had by now become clear) was separated into three parts.

The first part was adjusted to 0.2 M potassium phosphate–0.5 M KCl and irradiated. No new cross-link was detected.

The second part was directly eluted at low ionic strength on Sepharose 6B. All the repressor–oligonucleotide adducts were found to be complexed to DNA.

The third part was irradiated at low ionic strength before elution at high ionic strength. Between 45% and 50% of the adducts were found to be cross-linked again to the DNA.

These results show clearly that a repressor–oligonucleotide adduct can be complexed and cross-linked to DNA and consequently that there are at least two sites for nonoperator DNA binding and cross-linking on one repressor molecule (Figure 3).

Identification of the Cross-Linked Peptide. The procedure

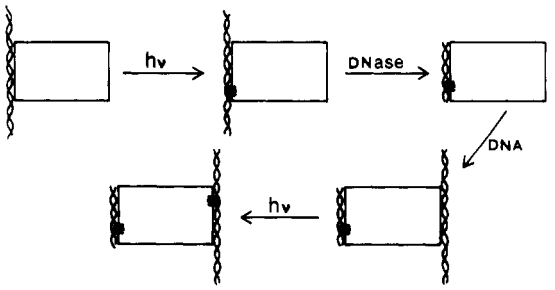

FIGURE 3: Reaction scheme for the two cross-linking sites experiment.

is described under Materials and Methods. Five different sets of results corresponding to five different preparations gave exploitable results, but with an important dispersion essentially due to the very small amounts of available material. Typically, the starting solution containing 10 mg of repressor and 1 mg of DNA contains in the best case 30 nmol of cross-linked peptides and 3 μmol of nucleotides. After all treatment steps (proteolysis, dialysis, gel filtration, hydrolysis, lyophilization, etc.), we have at our disposal only a few nanomoles of peptide linked to 100 times more nucleotide. Consequently, the determination of each amino acid in such small amounts (by difference with the DNA alone) does not allow precise measurements. Another cause of dispersion may result from variations in the kinetics of tryptic cleavage at the different Arg or Lys sites when the repressor is cross-linked. These changes could be sensitive to very small variations in the experimental procedure, and the mixture of peptides could exhibit some variations from one experiment to another.

The results of the amino acid analyses are shown in Figure 4. On the basis of these experimental data, a distribution of peptides (P_j) was computed for each experiment. The input values (see the Appendix and the A columns of Table II) were adjusted within the limits of experimental error to obtain a set of positive values for P_j. If this was not possible, a negative percentage up to 5% was considered as acceptable. The determined P_j values allowed us to compute the approximation values (B) (see the Appendix). To randomize the results, an average value for each amino acid was deduced from the expreimental data plotted in Figure 4A. Computation on the basis of these values gives the pattern showed in Figure 4B.

Discussion

The photochemical cross-link method appears very useful in identifying the regions of the interacting partners which are in contact in the complex. Nevertheless, if a positive reaction establishes that the residues are in contact, a negative one does not prove that there is no interaction. Among all the contact points between the two molecules, only those which can photochemically react are selected. From this point of view, we demonstrated that the headpieces are in close contact with the DNA but the absence of bridging with the core does not prove that it does not contact the DNA. Dunaway and coworkers (Dunaway et al., 1980) hypothesized that the core contacts the DNA only in the operator–repressor binding process, but not when the repressor is bound to nonoperator DNA. Unfortunately, our results cannot give further information about this point.

It is more surprising that we did not observe any cross-link between the isolated headpieces and the DNA, since the repressor is linked via the headpieces in the intact repressor. Considering that the binding of isolated headpieces induces similar changes in the CD spectrum of the DNA as the binding of intact repressor (Schnarr et al., 1983), it is highly probable that the conformational changes are in both cases quite similar.

Table I: Amino Acid Sequence for the First Six N-Terminal Tryptic Peptides of the Repressor (Farabaugh, 1978)[a]

j[b]	peptide sequence
1	Met-Lys-Pro-Val-Thr^{5}-Leu-Tyr-Asp-Val-Ala^{10}-Glu-Tyr-Ala-Gly-Val^{15}-Ser-Tyr-Gln-Thr-Val^{20}-Ser-Arg^{22}
2	Val-Val-Asn^{25}-Gln-Ala-Ser-His-Val^{30}-Ser-Ala-Lys^{33}
3	Thr-Arg^{35}
4	Glu-Lys^{37}
5	Val-Glu-Ala^{40}-Ala-Met-Ala-Glu-Leu^{45}-Asn-Tyr-Ile-Pro-Asn^{50}-Arg^{51}
6	Val-Ala-Gln-Gln^{55}-Leu-Ala-Gly-Lys^{59}

[a] Upon restricted conditions, peptides 1–51, 1–59, and 1–56 are isolated by using trypsin for 1–51 (short tryptic headpiece) and 1–59 (long tryptic headpiece) or chymotrypsin for 1–56 (chymotryptic headpiece). [b] The number j refers to the number of each peptide (see the Appendix).

Table II[a]

amino acid	i	expt 1 exptl[b]	1 A[c]	1 B[d]	2 exptl	2 A	2 B	3 exptl	3 A	3 B	4 exptl	4 A	4 B	5 exptl	5 A	5 B	av A	av B
Asx	1	11.2 ± 3.1	11.2	9.1	9.7 ± 1.5	9.7	8.3	11.8 ± 3.1	11.8	9.4	10.5 ± 6.0	10.5	9.2	14.1 ± 4.0	14.1	10.7	10.5	9.5
Thr	2	7.7 ± 2.3	7.7	6.9	7.7 ± 2.1	7.7	7.4	4.5 ± 4.5	8.0	5.1	5.0 ± 2.5	7.5	6.9	4.9 ± 1.8	4.9	3.7	6.0	5.6
Ser	3	11.8 ± 3.6	11.8	9.4	13.8 ± 2.0	13.8	10.0	14.2 ± 4.3	14.2	9.6	16.9 ± 10	12.0	10.5	10.0 ± 2.1	10.0	8.9	12.5	9.7
Glx	4	13.8 ± 2.0	13.8	13.4	13.8 ± 3.2	13.8	13.2	16.8 ± 2.1	16.8	15.1	11.3 ± 5.6	13.5	12.5	12.1 ± 2.0	14.1	14.2	14.0	13.5
Ala	5	16.3 ± 1.8	16.3	16.3	16.4 ± 3.6	16.4	16.1	17.9 ± 4.5	17.9	16.2	13.6 ± 5.0	16.6	15.4	16.8 ± 4.5	20.0	18.0	17.0	16.6
Val	6	16.5 ± 2.4	16.5	17.8	16.6 ± 2.3	16.6	18.8	13.5 ± 1.9	15.4	18.6	19.0 ± 5.0	19.0	18.1	17.3 ± 1.5	18.6	17.1	16.5	17.9
Met	7	0.3 ± 0.3	0.3	3.5	0.3 ± 0.3	0.3	3.1	3.3 ± 3.3	2.0	4.3	5.6 ± 3.1	3.0	2.8	2.2 ± 1.5	2.2	3.8	3.0	3.3
Ile	8	2.1 ± 1.3	2.1	2.2	1.4 ± 0.6	1.4	1.6	3.0 ± 3.0	1.5	2.3	1.2 ± 1.2	1.2	1.9	2.8 ± 1.2	2.8	3.1	1.6	2.3
Leu	9	5.2 ± 1.4	5.2	3.7	5.1 ± 0.7	5.1	3.7	5.1 ± 3.5	5.1	4.1	3.1 ± 1.9	3.1	2.3	5.1 ± 2.0	5.1	3.7	5.2	3.2
Lys	10	6.1 ± 1.1	6.1	6.2	5.8 ± 1.1	5.8	6.3	5.8 ± 2.0	5.8	6.6			7.3	8.0 ± 1.6	8.6	6.5	7.0	6.9
His	11	1.3 ± 1.0	1.3	3.3	1.1 ± 1.1	1.1	3.5	2.0 ± 2.0	2.0	2.7			4.4	0.9 ± 0.9	0.9	3.7	1.5	3.8
Arg	12	7.2 ± 0.9	7.2	7.8	7.3 ± 1.5	7.3	7.5	1.5 ± 1.5	3.0	5.4			8.0	5.8 ± 2.0	5.8	6.0	7.0	7.0

j	P	P	P	P	P	P
1	9.6	10.8	16.4	5.2	5.8	7.0
2	23.9	25.3	22.4	26.7	28.7	27.5
3	30.4	31.7	8.8	31.6	17.0	26.0
4	19.3	16.0	32.4	21.2	23.2	22.2
5	15.8	11.9	18.8	12.0	24.3	16.6
6	1.0	4.2	−1.3	−3.3	−1.1	−0.6

[a] All amino acid and peptide amounts are given as the percent of the total, except for experiment 4, where the total of the nine considered amino acids is normalized by the average value of the total of the nine corresponding amino acids in the four other experiments. [b] "exptl" corresponds to amino acid analysis of the fully digested cross-linked tryptic peptide, with the experimental error. [c] A is the input value for each amino acid given an acceptable solution set for P_j (lower part of the table). [d] From the P_j set of values, an approximation value B is computed for each amino acid.

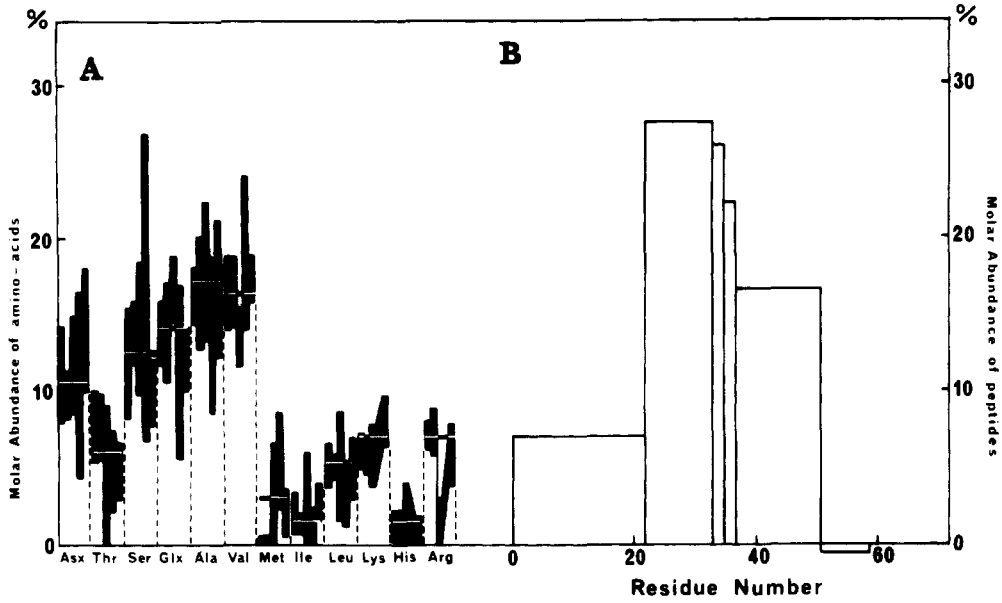

FIGURE 4: (A) Percent abundance of the 12 considered amino acids plotted for each of the 5 experiments. The white bars are the average values for each amino acid. (B) From the average values of each amino acid [white bars of (A)], a set of peptide abundances (P_j) is computed and plotted (as a histogram) vs. the length of each peptide. The area of the boxes is proportional to the mass of the peptide attached to the DNA. The height of the boxes is proportional to the number of each peptide cross-linked to the DNA (see Appendix for details).

Nevertheless, the differences in the photo-cross-linking behavior show that the interacting points of the headpiece are not exactly the same in both cases. This fact prompts us to consider the role of the core, not as a direct interacting partner but in maintaining an adequate relative position of the headpieces toward the DNA, more propitious to the interaction. The same conclusion concerning the specific interaction was inferred from operator–repressor and/or headpiece binding experiments (Culard et al., 1982).

The possibility of binding and of cross-linking DNA to a repressor already cross-linked to a short piece of DNA shows that there are two sites for nonspecific binding on the repressor. It has been demonstrated that two operator-bearing DNA fragments can bind the repressor (O'Gorman et al., 1980; Culard & Maurizot, 1981, 1982) but this fact was never previously demonstrated with nonoperator DNA. Winter & von Hippel (1981) reported unsuccessful attempts to observe the doubly bound repressor–DNA complex to test their "translocation model". Using 145 base pair fragments from chicken mononucleosomes (Charlier et al., 1983), we tried to form such complexes, without success. This could be due to the length of the fragments used, since negative charges of long fragments could induce a repulsive potential for the second fragment binding. In the experiments described here, the DNA was extensively digested, so that the DNA fragment was too short to maintain the adduct in solution. Consequently, the effect of negative charges in the vicinity of the second binding site was considerably limited, and the adduct can be resolubilized by binding to the added DNA.

Identification of the tryptic peptide bound to DNA cannot be made immediately since the analysis reveals the presence of more different amino acids than contained in one peptide. Examination of the computed peptide contributions (P_j) leads to the following remarks:

(1) A large dispersion appears in the contribution of peptides 3 and 4, but these peptides are dipeptides, and a small variation in the abundance of one of their components can induce a large variation in their calculated contribution.

(2) Peptide 6, which is considered as a hinge between the headpiece and the core, always appears to be less abundant, and its contribution is often found to be slightly negative.

(3) Among the three "long" peptides, peptide 2 is always the most abundant.

The nonunity of the cross-linked peptide could have the following two causes: (i) There are several possible cross-link points distributed over the headpiece. Since the first five peptides are found significantly in the analysis, all these peptides have at least one contact point with the DNA. The differences in their abundance may be a result of differences in the number of contact points and in the reactivity of these points. (ii) There is only one possible cross-link point on peptide 2, and, after the covalent bonding, the efficiency of the tryptic cleavage decreases and the digestion is not complete. It can be pointed out from Figure 3 that peptides 3, 4, and 5 remain relatively abundant. This could be due to the relative inaccessibility, after covalent bonding, of region 30–40, where three cleavage points are present.

A counterargument for the second hypothesis is the long duration of the tryptic digestion, first in native and then in denaturing conditions. Nevertheless, we shall consider this hypothesis as the most probable. Indeed, if numerous possible cross-linking points were distributed over all the tryptic peptides of the headpieces, it would be very surprising that, when the headpieces were isolated, any one of the points did not remain a possible cross-link point.

If this assumption is correct, the residue responsible for the cross-link belongs to peptide 2. All the amino acids present in peptide 2 are included in the 12 considered amino acids. Among them, only histidine shows an abnormal behavior. In the four experiments where it is present, the calculated concentration (B) is significantly greater than the experimental value, which never exceeds the background value. Histidine behaves as expected for an amino acid implicated in the cross-link. Another reason to consider histidine as a possible cross-linked residue is that it probably interacts with DNA, since it appears to be protonated and its pK shifts upon binding (Scheek et al., 1983; Schnarr et al., 1983). Lastly, a covalent bond between DNA and residue 29 would be very consistent with the hypothesis that positions 33, 35, and 37 are not as easily accessible as in the free repressor.

From the sequence homologies between the *lac* repressor and the cro repressor or the CAP protein, Matthews et al.

(1982) and Weber et al. (1982) proposed that the *lac* repressor interacts with DNA through an α helix. This helix would be extended from amino acids 17–26 and would contact the DNA in the major groove. Consequently, His-29 would be in the vicinity of the DNA, close enough to be cross-linked if the reaction is possible. Our results indeed confirm the fact that some interaction points of the DNA are situated in the major groove, where the methyl groups of the thymine and consequently the bromine atoms are located.

In conclusion, our results show that the photochemical cross-link of repressor to nonoperator BrU-substituted DNA occurs via the headpieces and that the core plays a fundamental role in this process by keeping the structure of the interacting headpieces in good fit with the DNA. The repressor has two sites for nonoperator DNA binding and cross-linking, and the cross-linked part of the headpiece is included in the second tryptic peptide (23–33); the contact point is presumably located at histidine-29. Work is now in progress to compare nonoperator and operator DNA, especially to elucidate the role of the core in the two processes.

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Appendix

After the cross-linked repressor has been digested by trypsin, the protein residue attached to DNA is hydrolyzed, and the amino acids are analyzed. The result is not representative of a unique peptide but can be approximated by a linear combination of the tryptic peptides. Let us call A_i the concentration of each of the N amino acids considered in our analysis (in our case, $N = 12$).

The sequences of the M possible peptides ($M = 6$ in our case) are known (Table I), and let us call α_{ij} the number of times peptide j contains amino acid i. The system is overdimensioned (i.e., $N > M$) and regular (i.e., no peptide is a linear combination of the others).

If P_j represents the concentration of each peptide j, we postulated the existence of a function B_i of P_j defined by

$$B_i = \sum_j \alpha_{ij} P_j \tag{1}$$

with

$$\sum_i (A_i - B_i)^2 \tag{2}$$

as a minimum.

With the use of matrix notations (a center dot represents the product and a bar the transposed form of a matrix), condition 2 will be fulfilled when

$$\frac{\partial}{\partial P_j} [\|\overline{A_i - B_i}\| \cdot \|A_i - B_i\|] = 0 \tag{3}$$

for a given value of $\|A_i\|$. In this equation, 0 represents a matrix line with the j terms equal to 0.

Carrying out the differentiation:

$$\frac{\partial}{\partial P_j} [\|\overline{A_i}\| \cdot \|A_i\|] - \frac{\partial}{\partial P_j} [\|\overline{A_i}\| \cdot \|B_i\| + \|\overline{B_i}\| \cdot \|A_i\|] + \frac{\partial}{\partial P_j} [\|\overline{B_i}\| \cdot \|B_i\|] = 0$$

For a given value of $\|A_i\|$, the first term is equal to zero and eq 3 becomes

$$\frac{\partial}{\partial P_j} [\|\overline{A_i}\| \cdot \|B_i\| + \|\overline{B_i}\| \cdot \|A_i\|] = \frac{\partial}{\partial P_j} [\|\overline{B_i}\| \cdot \|B_i\|] \tag{4}$$

As $\|\overline{A_i}\| \cdot \|B_i\|$ is a scalar, it is equal to its transposed form, and the first term can be written as follows:

$$2 \frac{\partial}{\partial P_j} [\|\overline{A_i}\| \cdot \|\alpha_{ij}\| \cdot \|P_j\|] = 2\|\overline{A_i}\| \cdot \|\alpha_{ij}\| \tag{5}$$

The second term of eq 4 may be expressed as follows:

$$\frac{\partial}{\partial P_j} [\|\overline{P_j}\| \cdot \|\overline{\alpha_{ij}}\| \cdot \|\alpha_{ij}\| \cdot \|P_j\|] = \overline{\|\alpha_{ij}\| \cdot \|\overline{\alpha_{ij}}\| \cdot \|P_j\|} + \|\overline{P_j}\| \cdot \|\overline{\alpha_{ij}}\| \cdot \|\alpha_{ij}\|$$

Let us call $\|Q\| = \|\overline{\alpha_{ij}}\| \cdot \|\alpha_{ij}\|$ a square regular symmetrical matrix of M lines and M columns. It is equal to its transposed form.

The second term becomes

$$2\|\overline{P_j}\| \cdot \|Q\| \tag{6}$$

Equalizing expressions 5 and 6 leads to

$$\|\overline{A_i}\| \cdot \|\alpha_{ij}\| = \|\overline{P_j}\| \cdot \|Q\| \tag{7a}$$

or

$$\|\overline{\alpha_{ij}}\| \cdot \|A_i\| = \|Q\| \cdot \|P_j\| \tag{7b}$$

$\|Q\|$ is a regular matrix. Equation 7a,b can be solved either by inverting $\|Q\|$ or by solving the system of M equations to M unknown values. The solutions for P_j allow one to calculate B_i by using eq 1.

In our case:

$$\|\alpha_{ij}\| = \begin{Vmatrix} 1 & 1 & 0 & 0 & 2 & 0 \\ 2 & 0 & 1 & 0 & 0 & 0 \\ 2 & 2 & 0 & 0 & 0 & 0 \\ 2 & 1 & 0 & 1 & 2 & 2 \\ 2 & 2 & 0 & 0 & 3 & 2 \\ 4 & 3 & 0 & 0 & 1 & 1 \\ 1 & 0 & 0 & 0 & 1 & 0 \\ 0 & 0 & 0 & 0 & 1 & 0 \\ 1 & 0 & 0 & 0 & 1 & 1 \\ 0 & 1 & 0 & 1 & 0 & 1 \\ 0 & 1 & 0 & 0 & 0 & 0 \\ 1 & 0 & 1 & 0 & 1 & 0 \end{Vmatrix} \qquad \|Q\| = \begin{Vmatrix} 36 & 23 & 2 & 2 & 19 & 13 \\ 23 & 21 & 0 & 2 & 13 & 10 \\ 3 & 0 & 2 & 0 & 1 & 0 \\ 2 & 2 & 0 & 2 & 2 & 3 \\ 19 & 13 & 1 & 2 & 22 & 12 \\ 13 & 10 & 0 & 3 & 12 & 11 \end{Vmatrix}$$

The experimental data used for the calculations are given in Table II.

Registry No. BrU, 51-20-7; histidine, 71-00-1.

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Deoxyribonucleic Acid Breaks Produced by 4′-(9-Acridinylamino)methanesulfon-*m*-anisidide and Copper†

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ABSTRACT: We have demonstrated that 4′-(9-acridinylamino)methanesulfon-*m*-anisidide (mAMSA), in the presence of Cu(II) ion, causes the breakage of plasmid pDPT275 and pBR322 superhelical form I DNA. In neutral pH, the degradative product was nicked, relaxed form II DNA, resulting from single-stranded DNA breakage. The extent of DNA breakage was both mAMSA concentration and Cu(II) concentration dependent. DNA breakage increased with increasing time of drug treatment. The mAMSA–Cu(II)-induced DNA breakage varied with pH values and also with the nature of the buffer systems. In both Tris-HCl and borate buffers the extent of DNA breakage increased with increasing pH. In Tris-HCl buffer (pH 7–9), only single-strand breaks were obtained, whereas in borate buffer (pH 9–10.5), linear form III DNA was obtained. At equivalent pH, the optimum buffer was borate. No breakage was observed at pH values below 6. The interaction of Cu(II) with mAMSA was examined by using absorption and fluorescence spectroscopies. Interaction of Cu(II) with mAMSA was characterized by a decrease in the absorption at 435 and 420 nm with a simultaneous increase at 330 nm. A highly fluorescent product was obtained upon reacting mAMSA with Cu(II), with an emission spectrum (excitation at 400 nm) showing a doublet at 430 and 450 nm and a shoulder around 480 nm. The spectral changes are also dependent similarly on the pH and the nature of buffer. Other divalent metal ions such as Co(II), Cd(II), Ni(II), and Zn(II) do not induce DNA breakage or spectral changes. The oAMSA isomer, which has no antitumor activity, is less effective in inducing DNA breakage than the mAMSA.

4′-(9-Acridinylamino)methanesulfon-*m*-anisidide (mAMSA)[1] is a promising synthetic anticancer agent. It is currently in phase II–III clinical evaluation. Clinical studies suggested that mAMSA is active in acute leukemia (Slevin et al., 1981). Its mechanism of action is still unclear, but the target seems to be DNA. mAMSA intercalates into DNA (Waring, 1976),

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[1] Abbreviations: dsb, double-strand break; ssb, single-strand break; EDTA, ethylenediaminetetraacetic acid; EB, ethidium bromide; mAMSA, 4′-(9-acridinylamino)methanesulfon-*m*-anisidide; mAQDI, N^1-(methylsulfonyl)-N^4-(9-acridinyl)-3-methoxy-2,5-cyclohexadiene-1,4-diimine; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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