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NONSPECIFIC BINDING OF *lac* REPRESSOR TO DNA

I. AN ABSORPTION AND CIRCULAR DICHROISM STUDY

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Nonspecific binding of *lac* repressor on DNA has been studied by absorption and circular dichroism (CD) spectroscopies. In a first step, the complex formation is accompanied by an absorption difference spectrum and a change of the CD signal of the DNA. The absorption difference spectrum is mainly due to a spectral change of the DNA. The variation of the CD signal has been analyzed according to a model calculation, which takes into account the fact that the excluded site is shorter than the perturbed site. We found that in this first step one repressor can bind every 14 ± 2 base-pairs, whereas one repressor perturbs 22 ± 2 base-pairs. In a second step, more repressor can bind on DNA, but without further change in the absorption and CD spectrum, indicating that another binding process occurs. The model calculation developed here is general for all binding processes inducing a perturbation over a length of DNA longer than that of the excluded site.

Introduction

The *lac* repressor plays an essential role in the *lac* operon regulation by binding to the *lac* operator sequence, thus preventing transcription of the *lac* structural genes. It has been observed that the repressor also binds to nonoperator DNA and polynucleotides with a considerably reduced affinity [1]. This binding, insensitive to the inducer, plays a role in operator-repressor recognition [2–5] and may be a 'precursor model' for specific binding studies.

The nonspecific binding has been extensively studied using DNA and synthetic polynucleotides, essentially poly[d(AT)]. Various methods were used to investigate the binding parameters of the complexation: nitrocellulose membrane filtration [1,6–10], ultracentrifugation [11,12], fluorescence [13,14], melting profiles [15,16], affinity chromatography [17–19], electron microscopy [20], CD [21–24] and light scattering [21]. There is general agreement on the fact that the binding constant is several orders of magnitude smaller than that for the operator binding and is strongly dependent on the ionic strength. However, large discrepancies are observed between the values reported for the length of the binding site, depending on the authors and also on the methods used. These lengths may be classified in two groups: long site lengths, determined by ANS-repressor fluorescence [13], melting curves [15] and electron microscopy [20], all ranging between 20 and 33 base-pairs; and short site lengths, which are observed by AENS-repressor fluorescence [14], melting curves [16], CD [23,24], light scattering [21] and ultracentrifugation [11,12], all ranging from 10 to 15 base-pairs. Zingsheim and co-workers [20] observed by electron microscopy that DNA coated with *lac* repressor looks like ribbons 200 Å in diameter and 70 Å

Abbreviations: AENS, *N*-acetylaminoethyl-1-naphthylamine-5-sulfonate; ANS, 8-anilino-1-naphthalenesulfonate.

high. These authors concluded that it was very likely that the repressor molecules are tightly packed on 'both sides' of the DNA helix. This result shows that care must be taken in the determination of the site length by dividing the length of the covered DNA by the number of bound proteins.

In most cases, the published results for the site length are expressed as a number of base-pairs. But they refer in fact to the ratio of the concentration of DNA to that of repressor when an observed parameter reaches a plateau. There is often confusion between the observed stoichiometry of the complex and the length of the site. This problem derives from the lack of a clear definition of the nonspecific site. Only one paper, by Butler and co-workers [23] has previously discussed this problem. We propose here a definition of the nonspecific site, based on topological considerations on the helical structure of DNA.

Another puzzling observation has been made: In spite of the fact that the *lac* repressor exhibits very low solubility at low ionic strength (e.g., in 10^{-3} M phosphate), we have observed that the binding of *lac* repressor to DNA allows solubilization of amounts of repressor several orders of magnitude larger than that for free repressor. Surprisingly, solubilization was observed even when the apparent saturation (as seen, for instance, by CD measurements) is overreached by a factor of two or three.

These facts prompted us to examine the nonspecific binding using two types of techniques in parallel. One is related to the DNA: absorption and CD spectroscopies (this paper). The other is related to the complex, and more essentially to the repressor: small-angle neutron scattering (following paper [39]). To interpret our CD data, we defined two types of sites: the steric site, independent of the investigation method, and the observed perturbed site, the 'dichroic site' in our case, directly related to the method used to follow the binding process.

2. Materials and methods

2.1. Biochemicals

The *lac* repressor was isolated according to Rosenberg and co-workers [28], from BMH 493 strain. The purity was checked by SDS-acrylamide gel electrophoresis. It was fully active with respect to the inducer and operator binding.

DNA fragments of 146 base-pairs were prepared from chicken blood mononucleosomes according to Libertini and Small [29]. The DNA was obtained after proteinase K and phenol treatments of mononucleosomes. The length of the fragments was determined using restriction fragments of definite length and was found to be 146 ± 4 base-pairs. Such short DNA pieces can be considered as rigid rods, with respect to their hydrodynamic properties [30].

The 'dialysed complexes' of DNA and repressor were prepared by mixing the two components at high ionic strength (0.2 M phosphate), followed by extensive dialysis against the binding buffer at 4°C for at least 48 h. The binding buffer used was 1 mM potassium phosphate, pH 7.25, and 0.1 mM dithiothreitol. This procedure ensures that the complexes are at the correct ionic strength. But, due to the dilution and the possible adsorption of material on the dialysis bags, it requires the determination of the final concentration of both components.

The 'addition complexes' were prepared by successive additions of small amounts of concentrated repressor to a solution of DNA. The DNA is in the binding buffer, but the repressor cannot be stored at this low ionic strength. The disadvantage of this procedure is that the ionic strength increases slightly during the titration (but in very reasonable limits: from 10^{-3} to 5.8×10^{-3} M phosphate). The obvious advantages are the rapidity and exact control of the concentrations of DNA and repressor.

2.2. Spectroscopy

Absorption spectra were recorded using a Beckman DU 8 spectrophotometer.

2.3. Ultraviolet difference absorption spectra

Difference absorption spectra were determined by measuring the absorbance of the complex and of the two components at discrete wavelengths. All absorbance values were in the range 0.4–1.2 (6–40% transmittance) in order to have the best accuracy. As the absorbing particles could have large molecular weights (up to 3.5×10^6 Da) the contribution of the scattered light to the absorbance cannot be neglected at the concentrations used (several mg/ml). According to Rayleigh's law [32], we considered that the absorbance excess due to the scattering was dependent on λ^{-4}. As the cell was removed from the apparatus for each repressor addition, the error due to poor geometrical positioning (reflexions on the cell walls, etc....) was considered independent of the wavelength, and the 'nonelectronic absorption' contribution to the absorbance was written as:

$$A_s(\lambda) = \beta + \frac{\alpha}{\lambda^4}$$

For one spectrum, β and α were determined using the absorbances at 350, 340, 330 and 320 nm, where we assumed that no electronic transition occurs for both components (and for the complex).

Known amounts of repressor stock solution were added (i) to the buffer or (ii) to the DNA solution. The absorbances at each wavelength were plotted as a function of repressor concentration. The difference absorption spectrum induced by complexation was thus deduced from the difference between the slopes in the first parts of the curves (fig. 1).

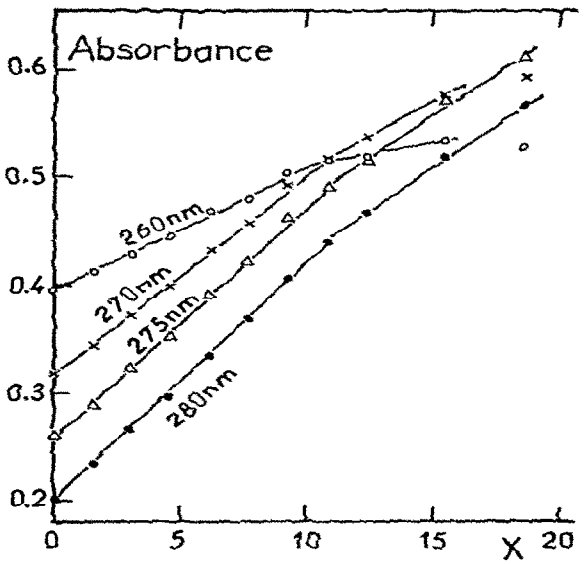

Fig. 1. Absorbance variations at four wavelengths of a DNA solution in which repressor is added. X is the ratio of repressor to DNA fragment. Straight lines are observed for $X < 10$. Their slopes give the absorption coefficient of complexes repressor. The difference absorption spectrum is obtained by comparison with the slopes of the curves obtained with repressor alone, at the same concentration.

2.4. Determination of the concentration of both components in the dialysed complexes

This step is essential in the study of the dialysed complexes. Let us call X the number of repressor molecules per DNA fragment. As discussed later, for $X \leqslant 10$, all the molecules of repressor are bound to the DNA in the first type of complex, exhibiting a difference absorption spectrum. For $X > 10$, the $(X - 10)$ molecules of the second type of complex do not induce any difference spectrum. Let us term $A(\lambda)$, $\epsilon_1(\lambda)$, $\epsilon_2(\lambda)$ and $\epsilon_3(\lambda)$ the absorbance of the solution and the molar absorption coefficients for the free DNA fragment, free repressor and difference spectrum, respectively, at a given wavelength λ. All these parameters are experimentally known (see fig. 3 for the values). For $X \leqslant 10$:

$$A(\lambda) = \epsilon_1(\lambda)C_1 + [\epsilon_2(\lambda) + \epsilon_3(\lambda)]C_2$$

For $X \geqslant 10$:

$$A(\lambda) = [\epsilon_1(\lambda) + 10\epsilon_3(\lambda)]C_1 + \epsilon_2(\lambda)C_2$$

C_1 and C_2 are the concentrations of DNA fragment and repressor, respectively. $A(\lambda)$ is corrected for the scattered light as previously described. The values of C_1 and C_2 and consequently $X = C_2/C_1$, are obtained by a least-square fitting over six wavelengths (290, 280, 275, 260 and 250 nm) of the $A(\lambda)$ values as a function of the ϵ values.

2.5. Circular dichroism measurements

The CD spectra were recorded from 250 to 340 nm using a Jobin Yvon Mark III dichrograph. The spectrum of the perturbed DNA was obtained from the difference between the measured spectrum of the complex and the contribution of the repressor, assuming that its dichroic spectra change in the aromatic region can be neglected as previously discussed [21]. The spectra were recorded at DNA concentrations ranging from 7×10^{-7} to 1.2×10^{-6} M fragment. We shall use the normalized CD difference signal at 275 nm as a representative parameter:

$$D = \frac{\Delta\epsilon - \Delta\epsilon_0}{\Delta\epsilon_\infty - \Delta\epsilon_0}$$

$\Delta\epsilon_0$, $\Delta\epsilon$ and $\Delta\epsilon_\infty$ are the dichroic signal per DNA fragment recorded in the absence of repressor, in the presence of repressor, and fully saturated by the repressor, respectively.

3. Analysis procedures

3.1. Tentative definition of the binding site

As the DNA lattice exhibits a repetition of potential sites for repressor binding, the definition of the site for nonspecific binding is often ambiguous. It is easier to define the specific site. Genetic engineering now makes possible the insertion of a given sequence of DNA in another DNA molecule, and we can propose from this fact a possible definition for the operator. Let us consider the operator region in *Escherichia coli* DNA. It includes the quasi-palindromic sequence described by Gilbert and Maxam [25] centered on a GC base-pair. The operator site can be defined as the smallest fragment of the *E. coli lac* genome centered at the GC base-pair mentioned before, which, when inserted in a long nonoperator DNA, gives rise to the same thermodynamic and structural properties towards the binding of the *lac* repressor as those of the wild-type operator region. This definition is essentially functional and does not depend on the method used for delimiting the site.

One can see immediately that such a definition cannot be extended to the nonspecific binding site. In most papers related to this problem, the binding site appears as a number of phosphate groups, bases, or base-pairs. But this number represents the concentration of one particular chemical function of the DNA, equivalent to the concentration of repressor when a parameter related to complex formation reaches a plateau. Except for Butler et al. [23], the problem of the spatial localization of these chemical groups was never discussed. One problem is that the covered length of DNA could be different from the sterically hindered length. Another point of confusion is that the DNA is often considered to be a uniformly charged cylinder, or worse, a planar ladder.

When one repressor binds the nonoperator DNA, we can assume that its position relative to the grooves and the phosphodiester backbones is absolutely defined. It is commonly proposed that two repressor subunits interact with the DNA (this fact is discussed in ref. 33). It is then likely that the pattern of contact points on the DNA has a C_2 axis of symmetry, which is the same than those of the two interacting subunits. This C_2 axis, which transforms one of the strands into the other one, is perpendicular to the DNA helix axis and bisects the two grooves in the middle cross-section of the interaction points pattern. Another repressor which would bind the DNA must find the same set of possible contact points in a position deduced from the first one by a helical transformation. This transformation is the product of a translation of kl, where k is an integer and l the distance between two lattice points, and a rotation around the DNA axis of $k\omega$, where ω is the angle between two base-plates. For example, in B-form DNA, l would be equal to 3.4 Å and ω would be equal to 36°. The smallest number, k, compatible with the steric restrictions and the induced conformational changes defines the excluded site, which does not depend on the investigation method used.

The observed site depends strongly on the technique used for observation. CD gives the average number of perturbed bases. Experiments using DNAse give the length of protected DNA and take into account the steric hindrance of the two proteins [25,37].

3.2. CD data analysis

We shall assume that the excluded site (i.e., the sterically hindered site) is k base-plates long.

The CD perturbed site is n base-plates long, and we shall assume that all the n base-plates are perturbed to the same average extent (fig. 2A).

Let us call $p = n - k$.

Overlapping of the excluded sites is forbidden, by definition. In the case $p > 0$, overlapping of the perturbed sites on $p/2$ base-plates of each side of the excluded site is possible. Nevertheless, we shall assume that the $p/2$ base-plates perturbed by the first repressor molecule do not exhibit further CD change upon binding of a second repressor molecule (fig. 2B and C).

The goal of this calculation is to establish a relationship between the number of repressors bound on the DNA and the fraction of perturbed base-plates of this DNA.

At the beginning of the binding process, the total perturbed length is proportional to the total hindered length (fig. 2D). If $p \leqslant 0$, this proportionality holds true over all the binding process. In the case of $p > 0$, overlapping of the perturbed sites destroys the proportionality between perturbed and hindered length when the total amount of repressor increases (fig. 2E).

We shall consider the DNA to be a very long chain of B base-plates, and we shall assume that the repressor molecules are randomly distribution along the DNA (i.e., there is neither cooperativity nor anticooperativity in the binding process, see the accompanying paper [39]). Let us term X the average number of repressors bound per DNA chain and $\nu = X/B$ the average number of repressors bound per base-plate.

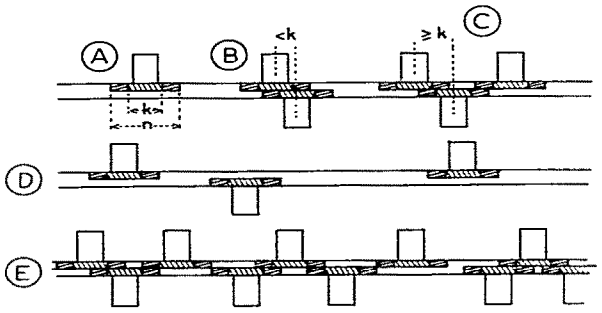

Fig. 2. Schematic drawing of the repressor, excluded site (k) and perturbed site (n) on the DNA. The repressors are represented alternately up and down for clarity.

The number of gaps (of 0 to B base-plates long) between two adjacent repressors is $(X + 1) \simeq X$ if X is large.

According to McGhee and Von Hippel [31] the probability of finding a gap of g free base-plates is:

$$P_g = \left(\frac{1 - k\nu}{1 - (k-1)\nu}\right)^g \left(\frac{\nu}{1 - (k-1)\nu}\right) \tag{1}$$

The number of gaps of length g is XP_g

The number of gaps of length greater than p is:

$$\sum_{g=p+1}^{B} XP_g \tag{2}$$

The number of perturbed base-plates in these gaps is:

$$P \sum_{g=p+1}^{B} XP_g \tag{3}$$

The number of gaps smaller than P is $\sum_{g=0}^{P} XP_g$ and they contain a number of perturbed base-plates equal to:

$$\sum_{g=0}^{p} XP_g g \tag{4}$$

The number of sterically hindered (and consequently perturbed) base-plates is:

$$Xk \tag{5}$$

The total number of perturbed base-plates is (eqs. 3–5):

$$Xk + \sum_{g=0}^{p} XP_g g + p \sum_{g=p+1}^{B} XP_g \tag{6}$$

The fraction of perturbed base-plates is:

$$F(k,p,\nu) = \nu\left\{k + \sum_{g=0}^{p} P_g g + p \sum_{g=p+1}^{B} P_g\right\}$$

Introducing the value of P_g (eq. 1) and summing the terms as a geometrical progression or its de-

rivative, we obtain, with B going to infinity:

$$F(k,p,\nu)=\nu\left\{k+\frac{1-k\nu}{\nu}\left[1-\left(\frac{1-k\nu}{1-(k-1)\nu}\right)^{p}\right]\right\} \qquad (7)$$

If we call D the normalized CD change (see above) averaged on the n perturbed base-plates: $D(k,p,\nu)\equiv F(k,p,\nu)$. Under our conditions, $\nu = X/146$, and $D(k,p,\nu)$ become $D(k,p,X)$.

Plotting D vs. X gives a curve which reaches a plateau ($D=1$) for $X=146/k$.

The slope of the tangent at the origin $\mathrm{d}D/\mathrm{d}X$ is equal to $(k+p)/146 = n/146$.

4. Results

4.1. Stability of the complexes

We measured the concentration of soluble repressor in our low ionic strength buffer by absorption spectrophotometry. It was found to be lower than 10^{-7} M (0.015 mg/ml). This value gives the maximum solubility of free repressor under our experimental conditions.

The dialysed complexes and the addition complexes are very stable if they are kept at 4°C. We observed neither precipitation nor change in the CD spectrum over a 1 week period, indicating that the concentration of free repressor at equilibrium is considerably lower than 0.015 mg/ml, the limit concentration ruled by the solubility. For this reason, we shall neglect it, considering that it is always very much smaller than the total concentration of repressor, which ranges from 0.12 to 6.4 mg/ml in our experiments. The measured ratio between the total repressor molecules and the DNA fragments present in the solution has been considered in our analysis to be equal to X, the average number of bound repressors per DNA fragment. We were able to prepare dialysed complexes with values of X as large as 24.

4.2. Absorption difference spectra

Fig. 3 shows the absorption difference spectrum for the binding of one molecule of repressor on a DNA fragment as deduced from the analysis previously described.

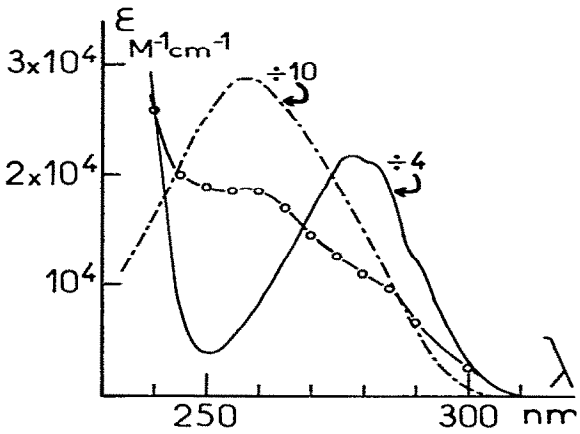

Fig. 3. Absorption spectra of the free repressor (———), a DNA fragment of 22 base-plates (- - - - - -), and the difference absorption spectrum for one repressor bound to DNA (O——— O). The circles are experimental values obtained at discrete wavelengths.

The measurements are possible with good accuracy for small values of X ($X<10$). For greater values of X, it is more difficult to interpret the changes in absorbance, due to the increase in scattering of the solution as the molecular weight of the particles increases. We cannot give an objective answer to the question: is there a difference absorption spectrum for the binding of repressor for $X \geqslant 10$? In any case, this difference would be small, and in our evaluation of X (see section 2.2) we assumed that for $X>10$, no difference spectrum was induced. This hypothesis is reinforced by the analogy with the CD effects, which are observable only for $X<10$.

4.3. Measurements

As previously shown, the addition of *lac* repressor to nonoperator DNA leads to an increase in intensity of the positive band of the CD signal of the DNA, in the wavelength range 250–300 nm [21–24]. This change of the CD signal has been attributed to a conformational change of the DNA. We have used the normalized CD change at 275 nm to monitor the binding process, as described in section 2. As shown in fig. 4, the slope of the

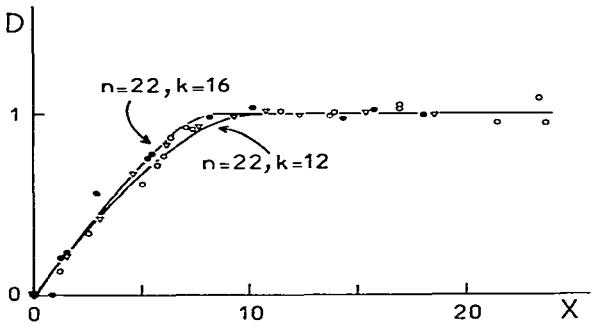

Fig. 4. Variation of the normalized CD signal of the DNA vs. the number of repressors bound per fragment. Three different experiments are represented: (○———○) and (●———●) represent two different experiments with dialysed complexes. (▽———▽) Experiment with the addition complex. The full lines are calculated from eq. 7 using the indicated parameters (see text).

tangent at the origin gives the extent of the perturbed site. We found $n = 22 \pm 2$ base-plates length. It is difficult to determine experimentally the value of k with good accuracy, since it is difficult to determine the point where the curve reaches the plateau. For this reason, we have looked for the best fit between the experimental data and theoretical curves calculated from eq. 7 with $n = 22$ and various values of k. The best fit was obtained for $k = 14 \pm 2$ base-plates.

A very surprizing result is that it is possible to obtain complexes containing more than one repressor per 14 base-pairs, without any further change in the CD signal. For these complexes, the total repressor concentration ranges from 1.5 to 3.9 mg/ml, i.e., 100- to 400-times greater than the maximum solubility of free repressor in our buffer. There is no doubt that all the repressor molecules bind the DNA, and we shall discuss in the following paper [39] a possible mechanism for this binding, which does not induce any detectable conformational change in the DNA.

5. Discussion

Our results clearly demonstrate that the binding of repressor to DNA induces an absorption difference spectrum. Such a difference spectrum is obtained for values of X smaller than 10. For values of X larger than 10, we cannot draw definite conclusions. The difference spectrum exhibits a maximum around 260 nm and a shoulder at 280 nm. From its shape, unambiguous assignment to DNA or to the repressor cannot be made. Changes in the absorption spectrum of the DNA are expected to produce a maximum effect around 260 nm, whereas changes in the protein should be observed around 280 nm. Since the CD signal of the DNA dramatically changes upon repressor binding as a result of a conformational change of the DNA, it may be assumed that the difference spectrum corresponds to a correlative change of the absorption of the DNA. In this assumption, the induced change of absorption per base is $\Delta\epsilon = 420\ M^{-1}$ at 260 nm, assuming also that the perturbation is uniformly distributed over the 22 base-plates of the perturbed site. Of course this value is an average value. Other contributions to the difference spectrum may not be negligible. The absorbance of the *lac* repressor might also change upon complexation with the DNA. It is now commonly admitted that the domains of the repressor which interact with the DNA are the N-terminal headpieces of the protomers [26,27,38]. These peptides do not contain any tryptophan, but they contain four tyrosines [34] as aromatic residues. In the case of a possible interaction of one or more tyrosine residues with the DNA, we cannot exclude a modification of these tyrosine absorption spectra (a shift, for instance) which could contribute to the absorption difference spectrum. In any case, the extent of the absorbance change per bound protein is of the same order of magnitude as the changes observed by other authors for DNA-RNA polymerase complexes [35,36]. However, in this latter case, it is known that the enzyme induces a melting of the DNA, whereas the change of CD spectrum absolutely excludes such a melting in the binding of *lac* repressor to DNA.

As previously noted [21,23,24], the change of the CD spectrum of the DNA upon repressor binding reflects a conformational change of the DNA. This conformational change is observed for values of X up to 10. Surprisingly, for values of

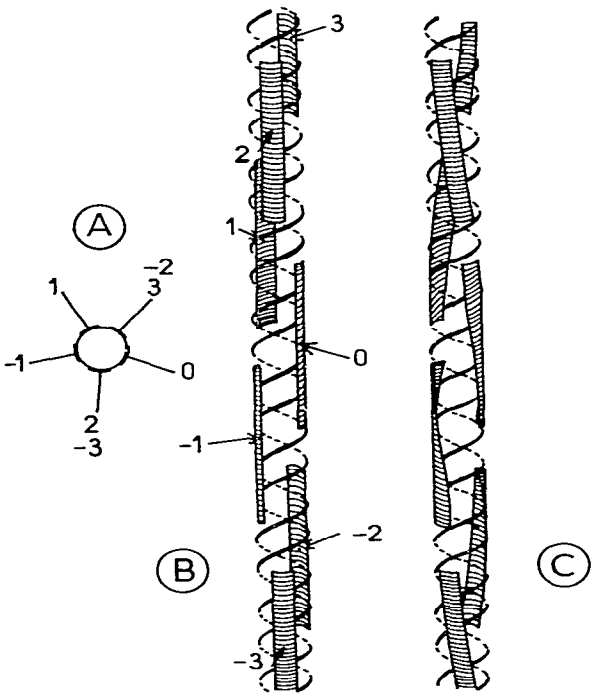

Fig. 5. Schematic drawing of the interacting sites for repressor-DNA complexes at saturation for the first type of complex. (A) Sectional view. (B and C) Side views showing the extent and overlapping of the perturbed sites. The arrows indicate the position of the C_2 axis (see text). In C, the surfaces of contact are drawn turned slightly to the left of the DNA axis, rather than parallel (see ref. 33).

$X > 10$, there is no more change of the CD signal and no precipitation of the 'excess' repressor. The simplest explanation is the existence of two types of complexes. The first one is accompanied by spectroscopic changes and is achieved when the DNA is covered by a first layer of repressor, corresponding to one repressor per 14 base-plates. As shown by neutron scattering experiments [39], the molecular weight of the complexes increases with X and is closely related to the average value of X. We can assume that in the second type of complex ($X > 10$), the repressors are bound to the first type of complex, but they do not induce any further CD change. The interpretation of this fact will be discussed in the following paper [39].

What is the significance of the length of the site we found? For the nonspecific binding, the value of $k = 14 \pm 2$ base-plates is the minimum distance at which we can find two nearest-neighbor repressors. It does not depend, as stated in section 3, on the investigation method used. It depends only on the nature of the binding site and on steric restrictions. Fig. 5 shows the possible arrangement of the repressor molecules along the DNA. It can be seen that the extent of the 'contact site' (i.e., the number of base-plates in contact with the repressor) can be larger than k. In fig. 5, we represent a contact surface 22 base-plates long, the same extent as the 'spectroscopically perturbed site'. However, it must be pointed out that the surface contact between the repressor and the DNA is probably not as shown in fig. 5B. If we refer to the surface contact involved in the specific binding [33], this surface is turned slightly to the left along the DNA axis, and fig. 5C is probably closer to reality, in very good agreement with the micrographs published by Zingsheim et al. [20] and with the assertion of Schmitz and Galas [37]: "At high repressor concentration, nonoperator DNA is protected completely from DNAse attack."

In the case of specific binding, the uniqueness of the site makes the definition of an excluded site meaningless. Consequently, care must be taken in the comparison between specific and nonspecific binding sites, since the first one is related only to n, which depends on the investigation method, whereas the latter may be related to n and/or to k, depending on the method.

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