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Lac repressor – *Lac* operator complexes

Solution X-ray scattering and electrophoretic studies

F. Culard[1], M. Charlier[1], J.-C. Maurizot[1]*, and A. Tardieu[2]

[1] Centre de Biophysique Moléculaire, C.N.R.S., 1 A, avenue de la Recherche Scientifique, F-45071 Orleans Cedex 2, France
[2] Centre de Génétique Moléculaire, C.N.R.S., F-91190 Gif-sur-Yvette, France

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Abstract. Complexes between the *Lac* repressor and a small DNA operator fragment (29 base pairs) were investigated using polyacrylamide gel electrophoresis and solution X-ray scattering. Titration of the DNA fragment with the repressor, followed by gel electrophoresis showed that only two types of complexes are formed with repressor/operator ratios of 0.5 and 2. Radii of gyration and forward scattered intensities were obtained from Guinier plots for repressor/operator ratios ranging from 0.3 to 2. They demonstrated that the first complex contains one repressor and two operators, whereas the second one contains four repressors and two operators. Mixing operator and repressor in equimolar concentrations leads to a mixture of both complexes. A possible model for the four repressor/two operator complex is proposed.

Key words: *Lac* repressor, *Lac* operator, X-ray scattering, electrophoresis

Introduction

The *Lac* repressor regulates the expression of the structural genes of the *Lac* operon in *E. coli.* The repressor binds with high affinity to the operator, a 20–30 base pair DNA sequence, and thus prevents transcription. Binding of the inducer (allolactose in vivo) to the repressor reduces the affinity of the latter for the operator sequence allowing the expression of the *Lac* gene (Müller-Hill 1975; Bourgeois and Pfahl 1976). The repressor also binds non-specifically to the rest of the *E. coli* genome with an affinity several orders of magnitude lower than that for the operator sequence. The ratio between the specific and non-specific affinity constants depends on the experimental conditions: pH, temperature, ionic strength, nature of the buffer and of the ions present in the medium. Under physiological conditions, this ratio is 10^8 (Lin and Riggs 1975; Revzin and von Hippel 1977; Record et al. 1977). Inducer does not affect repressor affinity for non-specific DNA sequences.

The *Lac* repressor is a tetrameric protein (4 × 360 a.a.) organized in domains, which may be cleaved by proteolysis. The four "headpieces" (a.a. 1–51 or 1–56 or 1–59, depending on the enzyme and the experimental conditions used) are the DNA (operator and non-operator) binding domains (Jovin et al. 1977; Ogata and Gilbert 1979; Nick et al. 1982a, b; Culard et al. 1982). The tetrameric "core" (4× a.a. 60–360) exhibits full inducer binding activity (Platt et al. 1973), and is thought to be implicated in the specific operator-repressor interaction (Matthews 1979; Dunnaway et al. 1980; Manly et al. 1983a, b).

Only few structural data relevant to the repressor are available, owing to the difficulty of growing single crystals suitable for X-ray crystallography. Electron microscopy and X-ray diffraction of microcrystals suggested an elongated shape for the repressor (Steitz et al. 1974). These authors and more recently Dunnaway et al. (1980), proposed an operator/repressor interaction model with the long axis of the repressor molecule parallel to the axis of the DNA helix.

More recent studies using small angle neutron scattering (Charlier et al. 1980, 1981) or X-ray scattering (McKay et al. 1982) confirmed the elongated shape of the repressor and the location of the headpieces about 60 Å from the centre of mass of the core. In models of operator binding proposed from theses studies, the long axis of the repressor is perpendicular to the DNA axis and two headpieces interact with the palindromic sequence of the operator.

In addition to these low resolution studies, NMR spectroscopy has been used to elaborate high resolution models for the headpiece (Zuiderweg et al. 1983, 1984; Kaptein et al. 1985) and for the head-

* To whom offprint requests should be sent

piece-operator interaction (Nick et al. 1982a, b; Buck et al. 1983; Scheek et al. 1983).

Despite the precise information obtained by these studies, since only the headpiece could be examined, complex formation with the entire repressor remains an open question. Even the possible stoichiometries are not unambiguously defined. We have therefore undertaken the study of these problems by non-denaturing polyacrylamide gel electrophoresis, and solution X-ray scattering. We used for that a short, 29 base pair, operator-DNA fragment and worked under relatively low ionic strength conditions.

The polyacrylamide gel electrophoresis method allowed us to visualise and separate free DNA from DNA-protein complexes, whose mobilities in polyacrylamide gel electrophoresis are related to both the molecular weight and the DNA/protein ratio (Garner and Revzin 1981; Fried and Crothers 1981; Crothers and Fried 1983; Kolb et al. 1983). The presence of two types of complexes, corresponding to two differents DNA/protein ratios (0.5 and 2), was demonstrated.

Solution X-ray scattering experiments were performed on these complexes, in order to study their structural arrangement. Titration experiments were also performed as a function of the repressor/operator ratio. The results are in agreement with a model for complex formation involving only the two types of complexes observed by gel electrophoresis, with no other intermediate species.

Material and methods

a) Preparation of the operator DNA

The *Lac* operator DNA fragment was isolated from a plasmid pBR345 (Bolivar et al. 1977), as described in Culard and Maurizot (1981). The fragment is a duplex of 25 base pairs, flanked on either side by four unpaired bases corresponding to the EcoR1 cuts

5′-AATTCA | AATTGTGAGCGGATAACAATT | TG-3′
3′-GT | TTAACACTCGCCTATTGTTAA | ACTTAA-5′

The boxed segment corresponds to the central 21 base pairs of the operator sequence (Gilbert and Maxam 1973).

We have previously shown (Culard and Maurizot 1981) that the fragment in solution assumes the classical B form of DNA. Polyacrylamide gel electrophoresis, as well as circular dichroism studies using the oligonucleotide AATT, have shown that there is no self-association by the cohesive ends under our experimental conditions.

Kallai et al. (1980), using a large scale method of preparation for the same 29 base pair fragment, reported the presence of single-stranded material. The single-strand DNA can adopt a hairpin conformation, due to the quasi palindromic sequence of the operator. The presence of the hairpin form is very easy to detect either by gel electrophoresis or circular dichroism. As shown on Fig. 1, our fragment preparations do not contain detectable amounts of hairpin form, which seems to occur only after particular conditions of denaturation/renaturation. As an illustration, we also show in Fig. 1 that heating the DNA sample to 53 °C, followed by rapid cooling to 0 °C leads, according to polyacrylamide gel electrophoresis, to more than 80% of the ethidium bromide fluorescence in the band corresponding to the hairpin form (Fig. 1A). The molecular percentage of hairpin form is probably even higher, as its affinity for ethidium bromide is lower than that of the duplex DNA. With circular dichroism, hairpin formation is also easily followed as the ratios of the intensities of the positive and negative bands are equal to 0.57 for the duplex and to 1.1 for the solution containing predominantly the hairpin (Fig. 1B). In our solutions, the absence of hairpin form was routinely checked by circular dichroism.

The four unpaired bases pairs at each end could be removed by nuclease S1 treatment, as decribed by Lillis et al. (1982). The progress of the digestion was checked by gel electrophoresis.

Fragment concentration was determined by spectrophotometric absorption, using a molar extinction coefficient $\varepsilon_{260} = 6{,}500\ M^{-1}\ cm^{-1}$ per nucleotide.

b) Preparation of the repressor

Wild type *Lac* repressor from strain BMH493 was purified according to Rosenberg et al. (1977), with the modifications mentioned in Culard and Maurizot (1981). Protein concentration was determined by spectrophotometric absorption, using a molar extinction coefficient $\varepsilon_{280} = 85{,}600\ M^{-1}\ cm^{-1}$.

c) Preparation of the complexes

The standard binding buffer used in all our experiments was 10 m*M* *Tris*-HCl, 10 m*M* KCl, 1 m*M* EDTA, 0.1 m*M* DTE, pH 7.5. (This buffer was suitable for both electrophoresis and X-ray scattering experiments.) Complexes were prepared either by direct mixing or by dialysis.

In the first case, small amounts of concentrated repressor (in 0.2 *M* potassium phosphate) were ad-

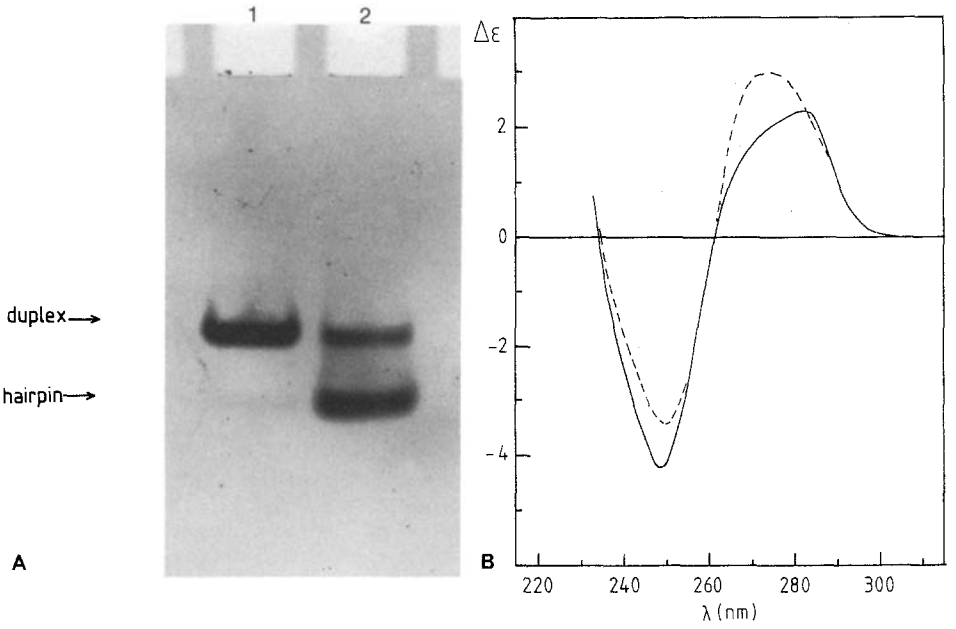


Fig. 1. **A** Electrophoretic patterns of native duplex operator fragment *(lane 1)* and the same fragment heated at 53 °C and quickly cooled in a ice bath *(lane 2)*. **B** Circular dichroic spectra of the native duplex operator fragment *(continuous line)* and the same fragment heated at 53 °C and quickly cooled in a ice bath *(dotted line)*. Experiments were performed in the standard binding buffer

ded to the DNA solution (in the standard buffer). This method was used for preparing low concentration solutions, since the ionic strength does not significantly vary upon repressor addition. In the second case, the DNA solution was lyophilised and the desired amount of repressor (in 0.2 *M* potassium phosphate) was added. After the DNA was redissolved, the solution was dialysed overnight in the cold room against the standard buffer. In this latter case, the absolute concentration of both components was redetermined after dialysis by fluorescence spectroscopy or/and by spectrophotometric absorption (Charlier and Maurizot 1983). We used this method to prepare more concentrated solutions (several mg/ml). When they were compared, both methods gave similar results.

Previous circular dichroism and gel filtration studies have shown that, in the concentration range used in the experiments described in this paper, our preparation of repressor is fully active (Culard and Maurizot 1981, 1982). These previous experiments, as well as the experiments described here, clearly show that there is no fraction of our *lac* operator DNA fragment which cannot bind the *lac* repressor.

d) Gel electrophoresis

Polyacrylamide gel slabs (140 × 160 × 3 mm, 5% acrylamide, 0.5% bisacrylamide) were cast in the standard buffer. They were run at 70 mA for 2 h. The complexes were prepared by direct mixing, and the amount of DNA was routinely equal to 0.5 μg per slot (corresponding to an operator concentration equal to 2×10^{-6} M). The DNA was first revealed using ethidium bromide fluorescence, then the proteins were stained with commassie brillant blue (Weber and Osborn 1972).

To perform autoradiographies, the DNA fragment was labelled with ^{32}P at the 5′ termini with polynucleotide kinase, after dephosphorylation with alkaline phosphatase (Maxam and Gilbert 1980). Autoradiograms were obtained by exposing Fuji X-ray films to the gels at − 20 °C. Complexation and gel electrophoresis were performed as for unlabelled fragments.

e) Low angle X-ray scattering

For these experiments, the complexes were prepared by dialysis. The concentration of repressor usually used ranged from 2 to 8 mg/ml (1.3 to 5×10^{-5} M).

The data were recorded with the small angle camera installed at the Synchrotron Radiation Laboratory, LURE (Orsay, France). The camera has been described in detail elsewhere (Koch et al. 1982). Briefly, the X-ray beam was monochromatized and focussed with a bent germanium crystal. The wavelength was 1.608 A, and a point collimation geometry was used. The detector was a linear position sensitive detector with delay line readout. Absolute scale measurements were obtained by reference to the scattering of a precalibrated carbon-black sample.

Table 1

	R	O	O_2R	OR	OR_2	O_2R_4
m (e) (a)	81,700	9,200	100,100	90,900	172,600	345,200
Mr (δ) (b)	152,400	17,800	188,000	170,200	322,600	645,200
V (Å^3) (c)	187,000	16,700	220,400	203,700	390,700	781,400
$\bar{\varrho}$ (e Å^{-3}) (d)	0.103	0.217	0.120	0.112	0.108	0.108
In(0)/Ce (e) (e)	4,530	1,430	7,010	5,750	10,270	20,540

(a, b) – The number of electrons m and the molecular weights Mr of each species are calculated from the sequences of the protein and of the DNA fragment. The ratios Mr/m we obtain are respectively 1.87 and 1.93 δ/e for protein and DNA.
(c) – The dry volumes V of the scattering particles are calculated from the volumes of the amino-acids. For the hydrophobic amino-acids Gly, Ala, Val, Leu, Ile, Phe, Met and Pro, the volumes per residue are from Chothia (1975). For the other amino-acids, the data are from Zamyatnin (1972). For the DNA, an average value of 577 Å^3 per base pair was calculated from the data quoted by Jacrot (1976). No volume variation due to the binding was considered. These values lead to partial specific volume $\bar{v}$ equal to 0.739 for the protein and 0.565 for the DNA.
(d) – The contrast $\bar{\varrho} = m/V - \varrho_0$, where ϱ_0 is the electronic density of the solvent, and is equal to 0.334 e Å^{-3} (water).
(e) – The forward scattered intensity $\mathrm{In}(0)/\mathrm{Ce} = m(1 - \varrho_0 V/m)^2 = (V\bar{\varrho})^2/m$. R and O are respectively the repressor and the operator fragment. O_nR_m symbolize complexes containing n operators and m repressors.

The data were recorded in different runs, with different preparations, with sample to detector distances varying from 80 cm to 1 m (ds/channel of the order of 1.5×10^{-4}, $s = 2 \sin\theta/\lambda$). The average exposure time was 10 min.

Samples were contained in quartz capillary tubes, about 1 mm in diameter.

Analysis of the data was made using Guinier plots, from which the radius of gyration and the forward scattered intensity (respectively Rg and In(0)/Ce, Luzzati and Tardieu (1980)) were determined.

Using the sequences of both operator and repressor, some parameters can be calculated for different types of complexes: number of electrons m, molecular weight Mr, dry volume V, partial specific volume $\bar{v}$, contrast $\bar{\varrho} = \varrho - \varrho_0$ (where ϱ is the mean electron density of the dry particle, and ϱ_0 the electron density of the solvant) and, from these values, the expected forward scattered intensity In(0)/Ce (Table 1). Note that only the product $\bar{\varrho} V$ is needed, which is independent of hydration. It was therefore calculated from the V and ϱ values relevant to a dry particle.

In the titration experiments, the evolution of the intensity at the origin and of the radius of gyration were modelled using the following formula, relevant to a mixture of species:

$$\mathrm{In}(0)/\mathrm{Ce} = \frac{\sum \mathrm{Ce}_i [\mathrm{In}_i(0)/\mathrm{Ce}_i]}{\sum \mathrm{Ce}_i} = \frac{\sum \alpha_i (\bar{\varrho}_i V_i)^2}{\sum \alpha_i m_i}, \quad (1)$$

$$\mathrm{Rg}^2 = \frac{\sum \mathrm{Ce}_i [\mathrm{In}_i(0)/\mathrm{Ce}_i] \mathrm{Rg}_i^2}{\sum \mathrm{Ce}_i [\mathrm{In}_i(0)/\mathrm{Ce}_i]} = \frac{\sum \alpha_i (\bar{\varrho}_i V_i)^2 \mathrm{Rg}_i^2}{\sum \alpha_i (\bar{\varrho}_i V_i)^2}, \quad (2)$$

where $\mathrm{In}_i(0)$ is the forward scattered intensity corresponding to the species i, Ce_i is the electronic concentration of the species i. Ce_i is proportional to the quantity $\alpha_i m_i$, were m_i and α_i are respectively the number of electrons and the molar fraction of the species i.

Rg_i, V_i and $\bar{\varrho}_i$ are respectively the radius of gyration, the volume and the contrast of each component i, and Rg, V and $\bar{\varrho}$ the corresponding parameters for the complex.

The molar fractions of each species i were calculated according to the pathway model explained in Results and shown in Table 2.

The distances d_i between the centre of mass of component i and the centre of mass of the complex can be calculated using the parallel axes theorem:

$$\mathrm{Rg}^2(\bar{\varrho} V) = \sum (\bar{\varrho}_i V_i) [\mathrm{Rg}_i^2 + d_i^2]. \quad (3)$$

Results

A) Electrophoretic study of the complexes

The electrophoretic behaviour of protein/fragment complexes, for repressor/operator ratios (X), equal to 0, 0.5, 1 and 2, is shown in Fig. 2. For $X = 0.5$, all of the DNA and protein are found in a single band which migrates differently from that of the free DNA. The existence of a complex containing one repressor for two operators has already been reported in previous studies (Culard and Maurizot 1981; O'Gorman et al. 1980a, b). For $X = 2$, there is

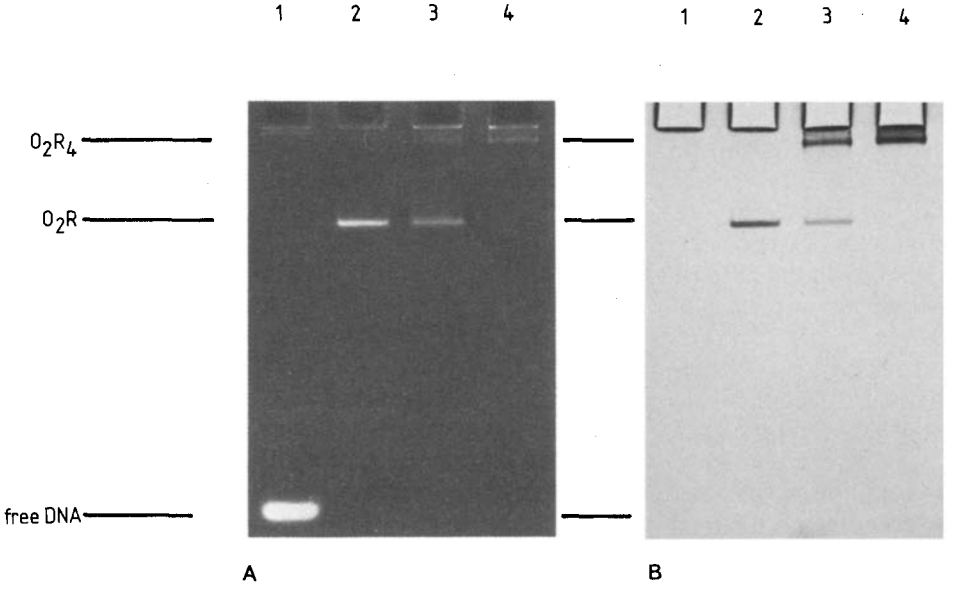


Fig. 2. Electrophoretic patterns of repressor-fragment complexes, for various ratios of X = repressor/fragment. **A** Ethidium bromide staining; **B** Coomassie blue staining. *Lanes 1:* $X = 0$ (fragment alone). *Lanes 2:* $X = 0.5$. *Lanes 3:* $X = 1$. *Lanes 4:* $X = 2$. The concentration of DNA fragment was 2×10^{-6} M, and the total DNA input was 0.5 µg per slot

Table 2. X is the molar ratio repressor/operator, and α_1, α_2, α_3 and α_4 are the molar fractions ($\sum \alpha_i = 1$) of the molecular species O, O_2R, OR and O_2R_4 respectively

Pathway 1	X		
	0	0.5	2
α_1	$\frac{1-2X}{1-X}$		
α_2	$\frac{X}{1-X}$	$\frac{4-2X}{3}$	
α_4		$\frac{2X-1}{3}$	

Pathway 2	X			
	0	0.5	1	2
α_1	$\frac{1-2X}{1-X}$			
α_2	$\frac{X}{1-X}$	$\frac{1-X}{X}$		
α_3		$\frac{2X-1}{X}$	$\frac{2X-4}{X-3}$	
α_4			$\frac{1-X}{X-3}$	

also a single band, with reduced electrophoretic mobility, compared to the previous case. In both cases, no free DNA fragment can be detected. For $X = 1$, we observed two bands, both containing DNA and protein, which migrate at the same position as the unique bands observed for $X = 0.5$ and $X = 2$ complexes.

In conditions where only one band of complex is observed ($X = 0.5$ and $X = 2$), we noticed that the intensity of the band, revealed by ethidium bromide fluorescence, is smaller than that of the free DNA. This might be due either to a loss of material or to a smaller degree of ethidium bromide binding to DNA in the complex. To decide between these two possibilities, electrophoresis was performed in similar conditions using ^{32}P labelled DNA fragments. After autoradiography, similar intensities were found in the bands of free DNA and both complexes. This clearly demonstrates a total recovery of DNA in the complex bands, and that the reduced fluorescence intensity of ethidium bromide in the complex bands is due to a smaller amount of dye bound to the DNA. Moreover, we observed that the complex band corresponding to $X = 0.5$ is more intense than that corresponding to $X = 2$.

These results suggest that titration of operator by repressor leads to the formation of two types of complexes: the first one contains one repressor for two operators, the second one contains two repressors for one operator. The presence of equivalent

concentrations of repressor and operator leads to a mixture of the two forms.

The operator fragment we used contained the two sticky ends resulting from the EcoR1 cleavage. We treated this fragment with nuclease S1, to obtain a 25 base pair fragment with blunt ends. Using this fragment, we could not detect any difference in the electrophoretic patterns of the complexes compared to those obtained with the 29 base pair fragment. We can thus exclude any influence of the cohesive ends on our results.

The effect of the inducer isopropyl-β-D-thiogalactoside (IPTG) was investigated. When the complex solutions were loaded on the gel in the presence of 1 m*M* IPTG, only the complex corresponding to one repressor for two operators could be dissociated. The complex containing two repressors for one operator could not be dissociated, even by increasing the ionic strength up to 0.15 *M* KCl.

B) Small angle X-ray scattering studies

Small angle X-ray scattering studies were performed on several samples with repressor/operator ratios ranging from 0.3 to 2. In each case, an electrophoresis was performed on an aliquot of the solution used for the scattering measurement. The only bands which have been detected were those corresponding to free fragment and to the two complexes mentioned in the previous paragraph. We did not detect any smearing on the gel either by ethidium bromide or by commassie blue staining.

For repressor/operator ratios between 0.3 and 2, the Guinier plots were always linear at low angles, and no trace of high molecular weight aggregates was ever observed. Two exemples are given in Fig. 3.

The values obtained for the normalized intensity at the origin when $X = 2$ (two repressors for one operator), were found reproducibly to be equal to 20,700 ± 400, very close to the values calculated for an O_2R_4 complex. The experimental values of In(0)/Ce for $X = 0.5$ although more disperse for a reason discussed later on, are in agreement with an O_2R complex.

Since under our experimental conditions the maximum amount of free repressor is very low, owing to its poor solubility in our buffer, and since we never observed any free DNA for $X > 0.5$, even on overloaded gels, we feel safe to assume that, for $X > 0.5$, all the material is complexed. This strongly suggests that the dissociation constants of both complexes are smaller than the concentrations we used.

These data, coupled to the gel electrophoresis results, provide strong arguments for the existence at

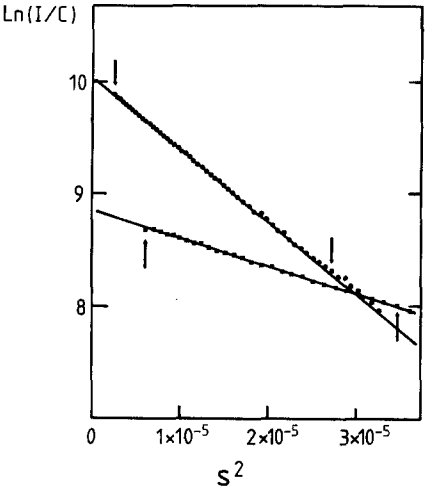


Fig. 3. Guinier plots of solutions containing mixtures of fragment and repressor, corresponding to $X = 0.5$ (protein concentration equal to 5.8 mg/ml, *lower curve*) and $X = 2$ (protein concentration equal to 7.5 mg/ml, *upper curve*). The arrows delimit the zone for calculating the straight line by linear regression

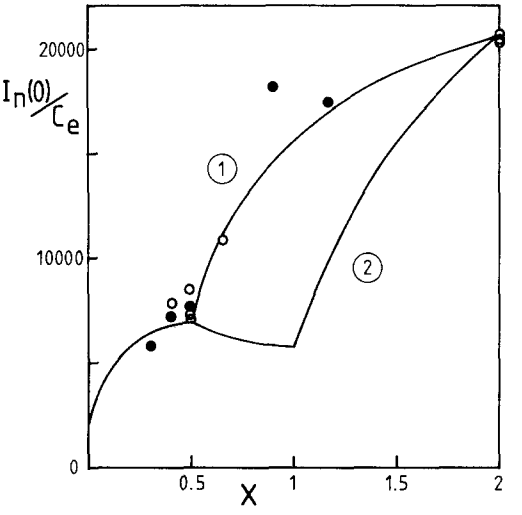
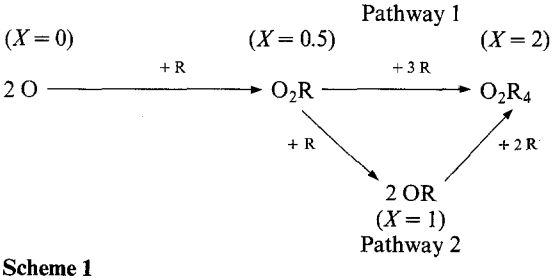


Fig. 4. Titration of the fragment with the repressor. In(0)/Ce is the forward scattered intensity, and X the ratio fragment/repressor. *Empty circles:* experiments performed on an absolute scale. *Full circles:* experiments performed on a relative scale and renormalized using the known value of In(0)/Ce for $X = 0.5$. The curves 1 and 2 correspond respectively to pathways 1 and 2 (see Scheme 1), and have been calculated using Eq. (1) from model data given in Table 1, and the molar ratios given in Table 2



Scheme 1

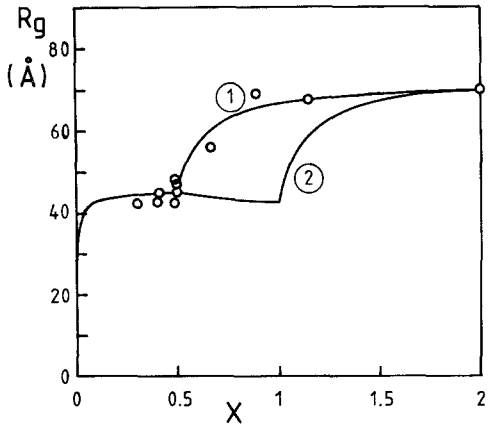


Fig. 5. Titration of the fragment with the repressor. Rg is the radius of gyration, and X the ratio fragment/repressor. The curves 1 and 2 correspond respectively to the pathways 1 and 2 (see Scheme 1). They have been calculated using Eq. (2), using the molar ratios given in Table 2, and the values of Rg respectively equal to 29 Å, 45 Å, 42.5 Å and 70 Å for the fragment alone and the complexes O_2R, OR (curve 2), and O_2R_4

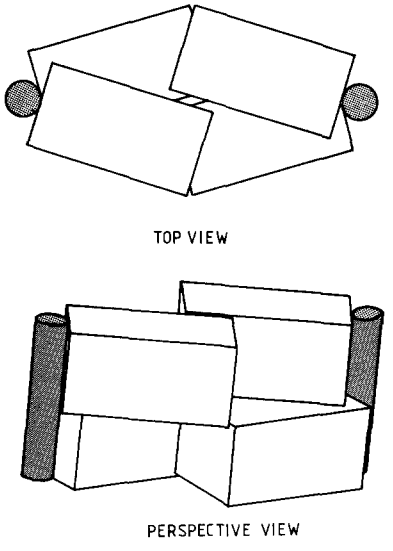


Fig. 6. Schematic drawing of a possible complex O_2R_4. The repressor has been represented as an elongated parallepiped of $50 \times 50 \times 100$ Å. The operator has been represented as a cylinder of 98 Å length and 20 Å diameter. This configuration is drawn for $d = 100$ Å and $L = 52$ Å, given a radius of gyration equal to 70 Å

$X = 0.5$ of an O_2R complex, and at $X = 2$, of an O_2R_4 complex.

We then performed a titration of the operator by the repressor, for values of X ranging from 0.3 to 2. The experimental values of In(0)/Ce and Rg deduced from the Guinier plots are shown on Figs. 4 and 5.

Using Eqs. (1) and (2), it is possible to calculate the titration curves In(0)/Ce and Rg as a function of X for various models of complex formation. Models were designed that make use of two different pathways, the first one where only O_2R and O_2R_4 are present and the second one in which a complex of intermediary stoichiometry, OR, may also be formed in the solution. The molar fractions α_i of each molecular species present in the solution as a function of X for both pathways 1 and 2 are given in Table 2. The calculated In(0)/Ce and Rg curves are shown in Figs. 4 and 5.

We cannot exclude the possibility that other models might fit the experimental data. However, the agreement of experimental data with data calculated according to pathway 1, the impossibility of fitting the data with pathway 2, coupled with the electrophoretic results, converge to indicate that complexation of operator to repressor does not involve formation of OR complexes. The singularity in the calculated In(0)/Ce and Rg curves at $X = 0.5$ also provide an easy explanation of why a small under/over estimation of X (determined by spectrophotometric absorption) may lead to a dispersion of the experimental values around $X = 0.5$.

C) Model design

O_2R_4 complex. It seems likely that, in the O_2R_4 complex, at least two of the four repressors cannot make specific interactions with the DNA. The total lack of IPTG effect suggests in addition that none of the four repressors binds to DNA through specific interactions and that the geometry of the interaction is more reminiscent of repressor binding to non-operator DNA, which is IPTG independent.

No particles of higher molecular weight are observed. A likely hypothesis is that the O_2R_4 complexes are formed by the association of the protein parts of two OR_2 particles, whose geometry is determined by the DNA fragment. Such an association would lead to a "closed" and symmetric complex and avoid the formation of larger particles.

As the radius of gyration of the repressor is known and equal to 40.5 Å (Charlier et al. 1980, 1981; McKay et al. 1982) and that of the operator fragment can be calculated to be 29 Å (assuming that it can be described by a rod of 3.4 Å per base pair, and 20 Å in diameter), it is possible, using Eq. (3), to estimate the distances between the centre of mass of the complex and each of its components. For the O_2R_4 complex, the radius of gyration depends upon six parameters. If we assume that the four repressor molecules are equidistant from the centre of mass (distance L) and that the two operator fragments are also equidistant from the centre of mass (distance d), the radius of gyration of the complex only depends upon two parameters, L

and d, which cannot be independantly determined. Putting in Eq. (3) the values for volumes and contrasts given in Table 1, and the values of the radii of gyration of the repressor and of the operator mentioned above, we obtain:

$$d^2 + 10.6 \times L^2 = 11.7 \times \mathrm{Rg}^2 - 18{,}300,$$

where Rg is the radius of gyration of the complex O_2R_4.

As Rg is equal to 70 Å (Fig. 5), L is not very sensitive to the value of d. For instance, the calculated values of L are equal to 60, 55 and 52 Å, for d equal to respectively 35, 80 and 100 Å. A tentative model of the O_2R_4 complex can be drawn from these values, as shown on Fig. 6.

O_2R complex. Assuming that the two operators are equidistant from the centre of mass of the complex (and consequently the repressor) that distance could, in principle, be determined using Eq. (3). However, we think our data does not allow us to make that calculation, as the dispersion of the Rg values for the O_2R complex is greater than that for the O_2R_4 complex (Fig. 5). Again, this dispersion is easily explained. A small underestimation of X would lead us to neglect a contamination of the O_2R complex by the O_2R_4 complex, whose contribution to the measured radius of gyration is important. Nevertheless, it is worth mentioning that models like those proposed in Charlier et al. (1981), are compatible with our experimental data.

Discussion

We have shown how two different techniques could be coupled to demonstrate the presence in solution of two types of operator/repressor complexes, O_2R and O_2R_4.

When considering the possibility of forming complexes such as O_2R and O_2R_4, we need to be sure that the material used is correctly defined. We have shown that our DNA operator fragment does not contain any detectable amount of hairpin form. We have also shown that the cohesive ends do not play any role in the complexation process, since digestion by nuclease S1 has no effect on the results obtained. Combination of these results with the fact that no free DNA can be detected by electrophoretic analysis of complexes for $X > 0.5$, shows that the operator is fully active. This assertion assumes that the repressor is 100% active, as indicated in Material and methods.

A complex containing two operators and one repressor had previously been observed by gel filtration and circular dichroism studies (Culard and Maurizot 1981), and by nitrocellulose filter binding assays (O'Gorman et al. 1980a). The presence of two binding sites on the repressor for non-operator DNA was reported by Barbier et al. (1984), using a photochemical crosslinking technique. The existence of such a double complex agrees very well with the models proposed for the DNA-repressor interaction (Dunnaway et al. 1980; Charlier et al. 1981; McKay et al. 1982). Previous experiments had shown that in this complex, the binding is specific. Our gel electrophoresis experiments confirm this point, since this complex dissociates when IPTG is added.

More surprising is the formation of O_2R_4 complexes and the absence of OR complexes. This absence could be linked to allosteric behavior of the protein. Since it is known that operator binding decreases the affinity for inducer, it is reasonable to suppose that the binding of the second operator could be positively dependent on the first one. However, the size of the DNA fragment is likely to play a role. Indeed, under the same experimental conditions, we have observed, by gel electrophoresis (Culard, unpublished results) both OR and O_2R complexes between repressor and an operator-bearing DNA fragment of 203 base pairs. One can imagine that for longer fragments, the electrostatic repulsion between DNA chains militates against the formation of O_2R and O_2R_4 complexes.

With regard to O_2R_4 complex formation, we can note that in low ionic strength buffer such as that used in the present work, the repressor is poorly soluble, owing to strong protein-protein interactions. Such interactions could stabilize the O_2R_4 complex. Similar results have already been obtained with non-operator DNA. Charlier et al. (1983) demonstrated, by small angle neutron scattering that DNA fragments from nucleosomes can be covered by more than one layer of repressor molecules. The second layer was in this case stabilized by protein-protein interactions.

In contrast to what is observed with O_2R complex, IPTG does not affect the O_2R_4 complex. This indicates that in this complex, the geometry of the interaction between the DNA and the protein is different from that which is found in the specific complex. This is not surprizing if one think that each operator fragment must contact at least two *lac* repressor molecules.

Do these complexes play a role in vivo? Several years ago, it was proposed that the *Lac* repressor could bind simultaneously the operator and a pseudo-operator sequence of the *E. coli* genome (Kania and Müller-Hill 1977). This could "enhance repression by keeping the *Lac* repressor, after its dissociation from the *Lac* operator, in the vicinity of the operator". Such a type of structure has been

more recently proposed in the case of the *Gal* operon (Irani et al. 1983). The *Gal* repressor, which is closely related to the *Lac* repressor (von Wilken-Bergmann and Müller-Hill 1982), has two binding sites on the *Gal* operon, and these two operators are involved in the repression of the *Gal* gene. The possibility for the *Lac* repressor to bind at the same time to two distinct sequences of DNA is needed in the "translocation model" proposed by Winter and von Hippel (1981), to explain the exceptionally high rate of complex formation between the *Lac* repressor and the *Lac* operator. These authors reported unsuccesful attempts to observe such doubly-bound repressor to operator fragments. The difference between their results and ours, may be explained by the fact that we used an operator sequence located on a short piece of DNA, and that we worked with solutions more concentrated in both repressor and operator.

There is no evidence that a complex such as O_2R_4 could play a role in vivo in the functioning of the *Lac* system. However, we think that its existence, as well as that of the O_2R complex, could be of considerable potential interest to those hoping to crystallize *Lac* operator-*Lac* repressor complexes as the first step towards high resolution structural studies. Our results show that, depending on the initial repressor/operator ratio, different types of complexes coexist, and especially that for a 1/1 ratio, a mixture of O_2R and O_2R_4 complexes might prevent any crystallization. It would clearly be advisable to investigate the types of complexes which are formed when mixing repressor and DNA fragments. It would for example be dangerous to assume that an equimolar solution of operator and repressor will lead necessarily to a 1/1 complex, whatever the experimental conditions.

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Note added in proof: Since this article was accepted, two publications (Besse M, von Wilcken-Bergmann B, Muller-Hill B (1986) EMBO J 5:1377–1381 and Mossing MC, Record T (1986) Science 233:889–892) have shown that a second *lac* operator introduced upstream or downstream from *lac* promoter, enhances repression and that a complex between *lac* repressor and DNA carrying two *lac* operators is exceedingly stable. These results further support the existence of the O_2R complex and its possible biological role.