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Stoichiometry of *lac* Repressor Binding to Nonspecific DNA: Three Different Complexes Form†

Robert C. Lawson, Jr.,‡ and Sheldon S. York*
Department of Chemistry, University of Denver, Denver, Colorado 80208
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ABSTRACT: The stoichiometry of *lac* repressor binding to nonspecific DNA was investigated by three different techniques. Four molecules of the fluorescent probe 5,5′-bis(8-anilino-1-naphthalenesulfonate) [bis(ANS)] bind to each repressor subunit with an average dissociation constant of 20 μM. Nonspecific DNA displaces most of this bound bis(ANS), reducing the fluorescence. Titrations of repressor with nonspecific DNA monitored with high [bis(ANS)] (5–15 μM) had end points at 8 base pairs per repressor. Lower [bis(ANS)] (0.1–1 μM) resulted in end points at either 15 or 26 base pairs per repressor, depending on the ionic strength. These end points correspond to complexes containing approximately one, two, or four repressors per 28 base pairs. Boundary sedimentation velocity experiments with saturating amounts of repressor revealed that five repressors can bind to 28 base pairs. By monitoring the circular dichroism as DNA was added to repressor, the sequential appearance of complexes containing approximately four, two, and one repressors per 28 base pairs was observed. The inability of repressor cores or iodinated repressor to bind to complexes containing one or two repressors per 28 base pairs implies that all of the repressors directly contact the DNA in the complex containing four repressors per 28 base pairs. It is proposed that while two subunits of each repressor contact the DNA in complexes containing one or two repressors per 28 base pairs, only one subunit of each repressor contacts the DNA in the complex with four repressors per 28 base pairs. These results suggest a novel mechanism for the one-dimensional diffusion of repressor along DNA.

The binding of the *lac* repressor both to its operator and to nonspecific DNA has been extensively studied and serves as a model for understanding specific protein–DNA interactions (Bourgeois & Pfahl, 1976; Muller-Hill, 1975; Wu et al., 1978). Many investigators have reported that the *lac* repressor binds to nonspecific DNA to form a complex containing two tetrameric repressor molecules per 28 base pairs of DNA, i.e., a complex with 14 base pairs per tetramer. This conclusion has been reached on the basis of circular dichroism studies (Butler et al., 1977; Durand & Maurizot, 1980), boundary sedimentation velocity studies (Revzin & von Hippel, 1977), thermal melting of the DNA in the presence of repressor (Wang et al., 1977), and studies with a fluorescent probe covalently bound to the repressor (Kelsey et al., 1979). A popular explanation for this result is that while each repressor spans 28 base pairs along the DNA, repressor molecules can bind along opposite sides of the same DNA segment, giving a complex with two tetramers per 28 base pairs. Zingsheim et al. (1977) have provided direct visual support for this model by observing a double layer of repressor tetramers bound to nonspecific DNA in electron micrographs. In contrast, Worah et al. (1978) have observed the formation of a complex containing only one repressor per 28 base pairs, using the fluorescent probe 8-anilino-1-naphthalenesulfonate (ANS).[1] This result implies that the first and second tetramers to bind per 28 base pairs are bound differently.

In this study, the stoichiometry of *lac* repressor binding to nonspecific DNA has been investigated in three different ways: by use of the fluorescent probe 5,5′-bis(8-anilino-1-naphthalenesulfonate) [bis(ANS)], by the boundary sedimentation velocity technique, and by circular dichroism. Bis(ANS), a covalent dimer of ANS (Farris et al., 1978), binds

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* Correspondence should be addressed to this author.
‡ Present address: Cell Technology, Inc., Boulder, CO 80301.

[1] Abbreviations: ANS, 8-anilino-1-naphthalenesulfonate; bis(ANS), 5,5′-bis(8-anilino-1-naphthalenesulfonate); CD, circular dichroism; Tris, tris(hydroxymethyl)aminomethane; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone.

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more tightly to the *lac* repressor than does ANS (York et al., 1978). Since a higher percentage of the bis(ANS) probe is bound to the protein, the fluorescence enhancement observed is greater than with ANS. When the repressor binds to nonspecific DNA, the fluorescence from this bis(ANS) probe decreases. This fluorescence decrease can be used to monitor the formation of protein–DNA complexes. The maximum number of repressors which complex with nonspecific DNA can be directly measured by using the boundary sedimentation velocity technique of Jensen and von Hippel (1977), by choosing conditions where the DNA is saturated with the repressor. The changes in the circular dichroism spectrum of nonspecific DNA when the repressor binds (Butler et al., 1977; Durand & Maurizot, 1980) make this another sensitive technique for monitoring repressor–DNA binding.

These different techniques have confirmed the existence of distinct complexes containing one or two repressor tetramers per 28 base pairs of DNA and have revealed the formation of a third complex, containing approximately four tetramers per 28 base pairs. A model is presented which accounts for the binding of four repressor tetramers per 28 base pairs. This structure suggests a novel manner in which the *lac* repressor might move along nonspecific DNA in its one-dimensional search for its operator.

Materials and Methods

Isolation of Repressor and Cores. The *lac* repressor was isolated from either *Escherichia coli* CSH 46 or *E. coli* pHIQ3. Growth of the CSH 46 (Worah et al., 1978) and pHIQ3 (Schneider et al., 1984) strains has been described previously. Repressor was purified by the method of Rosenberg et al. (1977) with modifications that have been described (Worah et al., 1978). A typical yield of purified *lac* repressor from 100 g of cell paste was 80 mg. The purity of the repressor, determined from sodium dodecyl sulfate–polyacrylamide gels (Miller, 1972), was ≥98%. These procedures yielded repressor which was approximately 50% active in operator binding, as assayed by a filter binding technique (Riggs et al., 1970). Repressor cores were prepared by the method of Geisler and Weber (1977) as described by Schneider et al. (1984), using TPCK–trypsin.

Poly[d(A-T)], with s_{20} values ranging from 7.2 to 10.7 S, was obtained from Miles Laboratories and P-L Biochemicals. Calf thymus DNA was obtained from Worthington. Stock solutions of DNA containing approximately 2 mg/mL were prepared in 0.1 M Tris-HCl, pH 8.0 at 25 °C, and 1 mM disodium ethylenediaminetetraacetate and stored at 4 °C. Calf thymus DNA was sheared by forcing a diluted solution through a 27-gauge needle (Davison, 1959). Dipotassium bis(ANS) was obtained from Regis and Molecular Probes.

The concentrations of the repressor and core proteins were determined from their absorbance at 280 nm by using the following ϵ_{280} values: *lac* repressor subunit, 22 125 M^{-1} cm^{-1} (Huston et al., 1974); *lac* core subunit, 18 240 M^{-1} cm^{-1} (Huston et al., 1974). The concentrations of bis(ANS) and DNA were determined from their absorbance by using the following values: bis(ANS), ϵ_{385} = 16 790 M^{-1} cm^{-1} (Farris et al., 1978); either poly[d(A-T)] or calf thymus DNA, $A_{260nm}^{1mg/mL}$ = 20 (Lin & Riggs, 1972).

Buffers. The 6 mM ionic strength buffer was 10 mM Tris-HCl, pH 8.0 at 25 °C, and 0.3 mM dithiothreitol. The 11 mM and higher ionic strength buffers were 20 mM Tris-HCl, pH 8.0 at 25 °C, and 0.3 mM dithiothreitol with NaCl added to obtain the desired ionic strength, except the 56 mM ionic strength buffer, which was 100 mM Tris-HCl, pH 8.0 at 25 °C, and 0.3 mM dithiothreitol. Just prior to use, stock solutions of *lac* repressor and repressor cores were thawed, diluted with the buffer being used in the experiments, and dialyzed 3–4 h against this buffer at 4 °C, with changes every hour.

Equilibrium Dialysis. Aliquots (0.5 mL) containing approximately 0.2 mg/mL repressor, with or without DNA, were placed in dialysis tubing. Bis(ANS) was added to each protein aliquot to adjust its initial concentration to that of the outside solution. Following dialysis for 12–18 h at 4 °C, the repressor solution was centrifuged for 30 min at 23000*g* to pellet any denatured protein, and the concentration of bis(ANS) was determined by absorbance and the concentration of repressor was determined by the method of Lowry et al. (1951) following precipitation by 8% trichloroacetic acid at 0 °C.

Fluorescence Measurements. All fluorescence measurements were made on a Perkin-Elmer MPF-3 fluorescence spectrophotometer, using a 4-mm path-length cuvette thermostated to 25 °C. Excitation and emission spectral bandwidths for experiments with bis(ANS) ranged from 4 to 7 nm. The stability of the fluorometer during the course of an experiment was monitored by using 1 μM quinine sulfate (Sigma) in 0.1 N H_2SO_4. Fluorescence spectra were not corrected for variations of the lamp intensity, photomultiplier response, or optical efficiency with wavelength.

In titrations of repressor–bis(ANS) mixtures with DNA, the protein concentrations ranged from 0.35 to 0.55 mg/mL. With high concentrations of bis(ANS) (5–15 μM), the excitation and emission wavelengths were 398 and 486 nm, respectively. With low concentrations of bis(ANS) (0.1–1 μM), the sample was excited with 360-nm light passed through a UV-D25 filter, and the emission was observed at 470 nm through a UV-43 filter, to minimize stray and scattered light. The fluorescence intensity of bis(ANS) complexed with repressor was corrected for inner filter effects, dilution effects, and, where necessary, contributions from repressor alone, as described by York et al. (1978).

Boundary Sedimentation Velocity Studies. According to the method of Jensen and von Hippel (1977), protein–DNA mixtures containing approximately 0.2 mg/mL repressor and 2.5–5.0 base pairs of DNA per tetramer were uniformly distributed throughout a 5–30% (v/v) glycerol (MCB Spectroquality) gradient and centrifuged at 50 000 rpm and 20 °C in a Beckman SW50.1 rotor for 1 h. Fractions (0.45 mL) were assayed for their protein and DNA concentrations. Repressor concentrations were determined from the tryptophan fluorescence, with excitation and emission wavelengths of 289 and 330 nm, respectively, using known concentrations in 5% glycerol as standards. DNA concentrations were determined by the method of LePecq and Paoletti (1966) using 5 μg/mL ethidium bromide and excitation and emission wavelengths of 540 and 580 nm, respectively.

Sedimentation of protein–DNA complexes through glycerol gradients was done as described by Worah et al. (1978). Centrifugation was for 45 min–1 h at 50 000 rpm in a Beckman SW50.1 rotor at 20 °C.

CD Studies. CD spectra and titrations were recorded on a Jasco J-500 spectropolarimeter at 20 ± 1 °C, using a 0.5-mL cuvette with a 1-cm path length. In a typical titration, aliquots of poly[d(A-T)] were added to 0.5 mL of solution containing 0.4 mg/mL repressor. The addition of 60 base pairs per tetramer to the 11 mM ionic strength buffer increased the ionic strength by 3 mM. The straight line segments in the plots of these titrations were fit by the method of least squares.

Iodination of Repressor. Iodination was done by the method of Fanning (1975). A solution containing 0.47 mg/mL re-

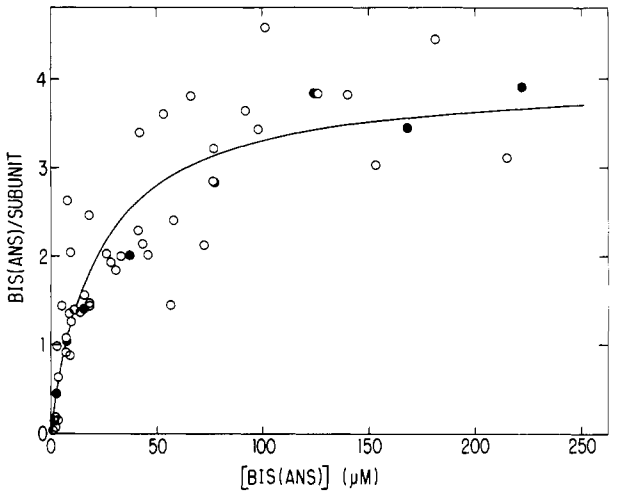

FIGURE 1: Equilibrium dialysis of repressor with bis(ANS). The number of bis(ANS) molecules bound per repressor subunit is plotted vs. the concentration of free bis(ANS) in the dialysis buffer, using the 56 mM (○) and 20 mM (●) ionic strength buffers at 4 °C. The best fit to the data at 56 mM ionic strength yielded a K_D = 22 μM and a maximum of 4.0 bis(ANS)/subunit and is shown as the solid line.

pressor, 2.2 μCi/mL $Na^{131}I$ (New England Nuclear, carrier free), 80 μM I_2, and 0.32 mM KI in 0.04 M Tris-HCl, pH 7.5 at 25 °C, 0.2 M KCl, 0.01 M $Mg(OAc)_2$, and 5% glycerol was allowed to react for 1 min at 4 °C. The reaction was terminated by adding excess 2-mercaptoethanol. Following dialysis, the amount of iodine incorporated was determined by its radioactivity, and the concentration of repressor by absorbance. The original reaction solution was prepared by mixing equal volumes of protein and KI_3 solutions. The KI_3 solution was prepared by adding $Na^{131}I$ and then a stock solution of KI_3 containing 0.01 M I_2 and 0.04 M KI to the reaction buffer. The equilibration of ^{131}I with the I_2 was ascertained by extraction with CCl_4.

RESULTS

Binding of Bis(ANS) to Repressor. The fluorescent probe bis(ANS) binds noncovalently to the *lac* repressor. The results of equilibrium dialysis experiments in the absence of DNA are shown in Figure 1. Four bis(ANS) molecules bind per repressor subunit with an average dissociation constant of 2 × 10^{-5} M. Essentially the same result was obtained at ionic strengths of 20 and 56 mM. While the different binding sites for bis(ANS) probably display heterogeneous binding behavior, the data in Figure 1 do not permit a more detailed analysis. Schneider et al. (1984) have presented evidence indicating that these bis(ANS) molecules bind to the interface of the core and headpiece regions of the repressor.

Equilibrium dialysis experiments in the presence of 45–60 base pairs of poly[d(A-T)] per repressor tetramer yielded quite different results (Figure 2). At concentrations of bis(ANS) below 20 μM, the binding of bis(ANS) is significantly reduced. Above 20 μM bis(ANS), the number of molecules bound increases sharply, reaching the same levels seen in the absence of DNA above 100 μM bis(ANS). Essentially the same behavior was seen at 20 and 56 mM ionic strength. Figure 2 presents data only to 90 μM bis(ANS) to emphasize the region of the binding curve most influenced by DNA.

These results suggest that bis(ANS) and poly[d(A-T)] compete with each other for binding to the repressor. This was directly shown in centrifugation experiments. Boundary sedimentation velocity experiments (see below) in the presence of 10 μM bis(ANS) confirmed that this concentration of bis(ANS) did not interfere with stoichiometric binding of

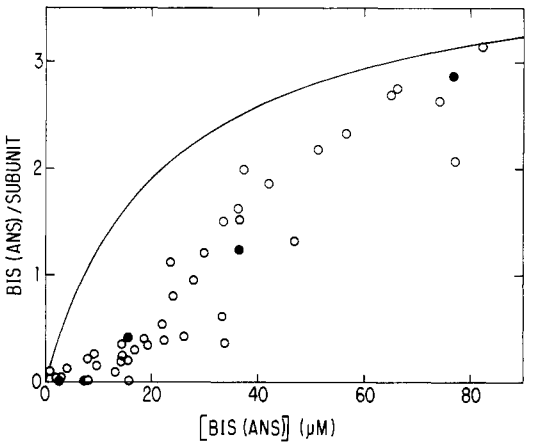

FIGURE 2: Equilibrium dialysis of repressor with bis(ANS) in the presence of poly[d(A-T)]. The number of bis(ANS) molecules bound per repressor subunit is plotted vs. the concentration of free bis(ANS) in the dialysis buffer, using the 56 mM (○) and 20 mM (●) ionic strength buffers at 4 °C. Poly[d(A-T)] was present at 45–60 base pairs per tetramer. The solid line is the best fit to data in the absence of poly[d(A-T)], taken from Figure 1.

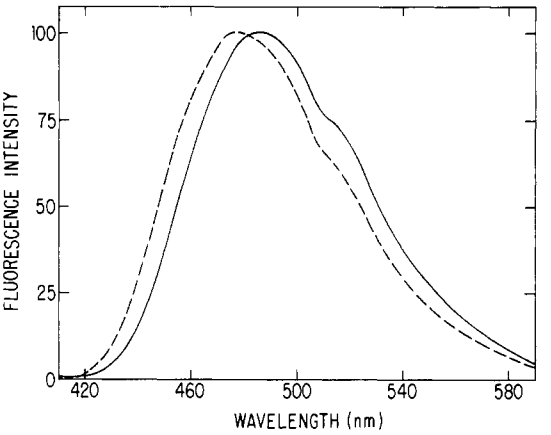

FIGURE 3: Fluorescence emission spectra of bis(ANS) bound to repressor recorded with 0.8 (– –) and 10 (—) μM bis(ANS) in the presence of 0.33 mg/mL repressor, using the 20 mM ionic strength buffer, at 25 °C. The maximum intensity of each spectrum has been normalized to 100. The spectrum with 0.8 μM bis(ANS) was corrected by subtracting the spectrum of repressor alone, which contributed 2% of the fluorescence intensity at the emission maximum. The contribution from repressor alone was negligible at 10 μM bis(ANS). The excitation wavelengths were 396 (– –) and 397 (—) nm, respectively, which were the maxima of the corresponding excitation spectra.

repressor to poly[d(A-T)]. Zonal centrifugation of a repressor–poly[d(A-T)] mixture containing 15 base pairs per tetramer in the presence of 200 μM bis(ANS), through a 5–30% glycerol gradient in the 56 mM ionic strength buffer which was also 200 μM bis(ANS), showed that no binding of repressor to poly[d(A-T)] had occurred. Under these conditions in the absence of bis(ANS), greater than 90% of the repressor complexes with poly[d(A-T)] of this size and moves away from the top of the gradient (Worah et al., 1978). In the experiments to follow, bis(ANS) was used to monitor the binding of repressor to poly[d(A-T)] and calf thymus DNA. The concentration of bis(ANS) was kept below 20 μM, allowing the repressor to bind stoichiometrically to the DNA under the conditions employed.

Fluorescence of Bis(ANS) Bound to Repressor. The fluorescence emission from bis(ANS) is greatly increased by binding to the *lac* repressor. Figure 3 shows the emission spectra of 0.8 and 10 μM bis(ANS) in the presence of repressor. In the absence of repressor, the emission intensities

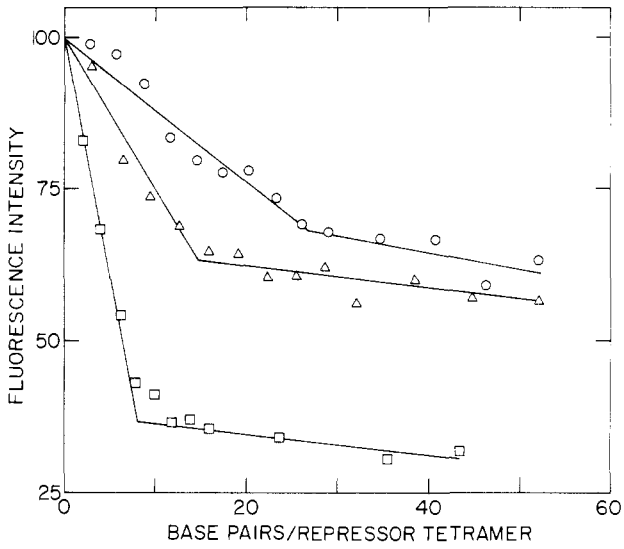

FIGURE 4: Titrations of repressor–bis(ANS) mixtures with poly[d-(A-T)] with 0.5 μM bis(ANS) and 30 mM ionic strength buffer (○), with 0.5 μM bis(ANS) and 20 mM ionic strength buffer (△), and with 10 μM bis(ANS) and 20 mM ionic strength buffer (□). With 0.5 μM bis(ANS), the sample was excited with 360-nm light passed through a UV-D25 filter, and emission was observed at 470 nm through a UV-43 filter. With 10 μM bis(ANS), the excitation and emission wavelengths were 398 and 486 nm, respectively. The initial intensity of each titration has been normalized to 100.

were only 1% those observed in the presence of repressor. The emission maxima at these low and high concentrations of bis(ANS) differ by 9 nm, being 477 nm at 0.80 μM and 486 nm at 10 μM bis(ANS). This implies that the repressor possesses at least two different types of bis(ANS) binding sites. At low concentrations of bis(ANS), one type of site is preferentially populated, while at higher concentrations of bis(ANS) additional types of sites are also populated. This result is not surprising considering that four molecules of bis(ANS) can bind to each subunit. The emission maximum of bis(ANS) in water is 520 nm. The blue shifts observed upon binding to the repressor imply that the environments surrounding this probe when bound to the protein are less polar than water, with the sites populated at low concentrations of bis(ANS) being less polar than the additional sites populated at higher bis(ANS) concentrations.

Repressor–DNA Binding Monitored by Bis(ANS). The fluorescence emission from bis(ANS) bound to repressor decreases as the repressor complexes with nonspecific DNA. This decrease can be used to monitor the stoichiometry of complex formation. Figure 4 shows three titrations of repressor–bis(ANS) mixtures with poly[d(A-T)], where the concentration of bis(ANS) and/or the ionic strength have been varied. With 10 μM bis(ANS) and 20 mM ionic strength, an end point was seen at 8.0 base pairs of poly[d(A-T)] per repressor tetramer. When the concentration of bis(ANS) was decreased to 0.50 μM, the end point increased to 15 base pairs per tetramer. Increasing the ionic strength from 20 to 30 mM while holding [bis(ANS)] at 0.50 μM further increased the end point to 27 base pairs per tetramer.

Since the amount of DNA required to reach an end point varied greatly as the conditions of the titration were changed, the effects of varying [bis(ANS)] and ionic strength on these end points were explored. Figure 5 shows the effect of changing the concentration of bis(ANS) as the ionic strength was held constant at 11 mM. The end points were restricted to two plateau values. At low concentrations of bis(ANS) (0.1–1 μM), end points of approximately 15 base pairs per

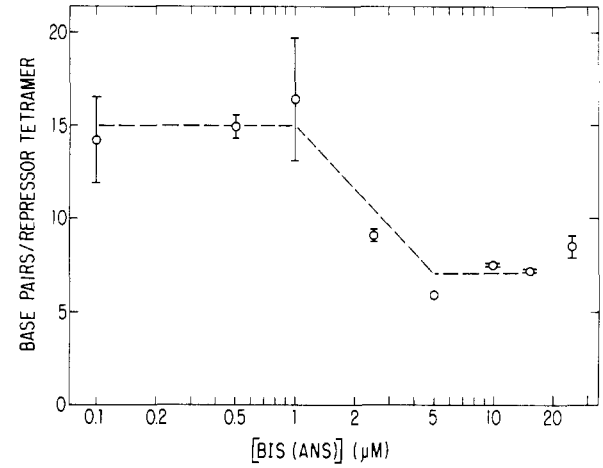

FIGURE 5: End points in titrations of repressor with poly[d(A-T)] monitored with different bis(ANS) concentrations. All titrations were in the 11 mM ionic strength buffer. Mean ± SD values are given. The plateau at 15.0 base pairs/tetramer is the average of values at 0.1–1.0 μM bis(ANS). The plateau at 7.1 base pairs/tetramer is the average of values at 5–15 μM bis(ANS).

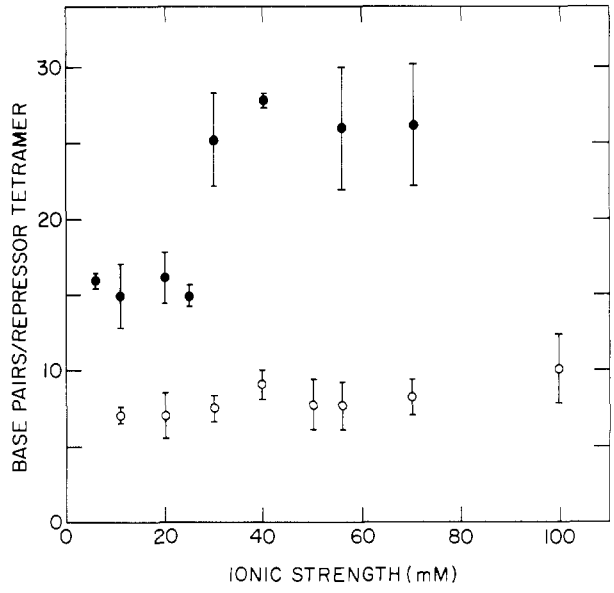

FIGURE 6: End points in titrations of repressor–bis(ANS) mixtures with poly[d(A-T)] at different ionic strengths. Titrations were carried out with high (5–15 μM) (○) and low (0.1–1.0 μM) (●) concentrations of bis(ANS). Mean ± SD values are given.

tetramer were observed, while at high concentrations of bis(ANS) (5–15 μM), the end points shifted to approximately 7 base pairs per tetramer. Apparently, these low and high concentrations of bis(ANS) are reporting the formation of different repressor–DNA complexes. An analogous situation was seen at 56 mM ionic strength, except now the plateau at low concentrations of bis(ANS) was found at approximately 26 base pairs per tetramer.

The effect ionic strength has on the titration end points is shown in Figure 6. Titrations were carried out both at high (5–15 μM) and at low (0.1–1 μM) concentrations of bis(ANS). At high concentrations of bis(ANS), a single end point was seen from 11 to 70 mM ionic strength, the average of 45 titrations being 7.8 ± 1.3 base pairs per tetramer. The end points of titrations carried out at 100 mM ionic strength were not as distinct as those at lower ionic strengths, and the average end point, 10 ± 2 base pairs per tetramer, was somewhat higher. O'Gorman et al. (1980) have reported a binding site size of 11.5 base pairs per tetramer at 0.11 M ionic

Table I: Stoichiometry of the Repressor–Poly[d(A-T)] Complex Observed in Boundary Sedimentation Velocity Experiments

ionic strength (mM)	base pairs per tetramer: before sedimentation[a]	base pairs per tetramer: in sedimenting complex[b]	free repressor (μg/mL)[c]
6	2.5	5.1 ± 0.8	115 ± 8
	5.0	6.4 ± 2.1	30 ± 10
20	2.5	4.8 ± 1.6	99 ● 9
	5.0	5.0 ± 0.2	38 ± 17
56	2.5	6.0 ± 1.3	145 ± 5
	5.0	5.6 ± 1.9	75 ± 10

[a] Initially present throughout tube before centrifugation begun. [b] Obtained from (slope)$^{-1}$ of repressor vs. poly[d(A-T)] plots. [c] Obtained from y intercept of repressor vs. poly[d(A-T)] plots.

strength, based on boundary sedimentation velocity experiments. The end points from these fluorescence titrations are in good agreement with this value. At low concentrations of bis(ANS), two different end points were observed, depending on the ionic strength. From 6 to 25 mM ionic strength, the average end point from 17 titrations was 15.4 ± 1.8 base pairs per tetramer, while from 30 to 70 mM ionic strength, the average end point from 20 titrations was 26.0 ± 3.4 base pairs per tetramer. Titrations done with sheared calf thymus DNA at high bis(ANS) concentrations gave the same end points as obtained with poly[d(A-T)]. With 10 μM bis(ANS) and ionic strengths of 30, 56, and 70 mM, end points of 6.8 ± 0.5, 6.7 ± 0.2, and 8.4 ± 2.0 base pairs per tetramer, respectively, were obtained. These titrations, under varying conditions of [bis(ANS)] and ionic strength, have revealed the existence of three different repressor–DNA complexes, with approximate stoichiometries of one, two, and four repressor tetramers per 28 base pairs of DNA, i.e., 28, 14, and 7 base pairs per tetramer, respectively.

The fluorescence decrease in titrations at high concentrations of bis(ANS) was greater than with low concentrations (Figure 4). The decrease at high bis(ANS) concentrations, 60 ± 11%, was quite constant from 11 to 56 mM ionic strength. At low bis(ANS) concentrations, the decrease was 34 ± 15%, varying little from 6 to 56 mM ionic strength. It is interesting to note that while the end point in titrations with low concentrations of bis(ANS) abruptly changes between 25 and 30 mM ionic strength, the total decrease in fluorescence at the end point remains quite constant.

Boundary Sedimentation Velocity Studies. The stoichiometry of the complex formed between excess repressor and poly[d(A-T)] was directly measured by using a boundary sedimentation velocity technique (Jensen & von Hippel, 1977). Glycerol gradients containing a uniform concentration of repressor and DNA throughout were sedimented, creating a boundary of sedimenting protein–DNA complex. At the concentrations of protein and DNA and the ionic strengths used in these studies, the binding of repressor and DNA was stoichiometric. Since repressor was in excess, the DNA was saturated with protein.

Figure 7 shows the results of one such experiment. By plotting DNA vs. protein concentrations of fractions in the boundary region, a linear plot was obtained (Figure 7, inset). The slope of this plot yields the amount of repressor bound to the DNA, while the y intercept yields the concentration of free repressor present throughout the gradient. The results of many such experiments are summarized in Table I. The average number of base pairs per tetramer in the complexes formed in 19 separate experiments was 5.5 ± 1.3, or approximately 5 tetramers per 28 base pairs. The same result was obtained at ionic strengths ranging from 6 to 56 mM, and

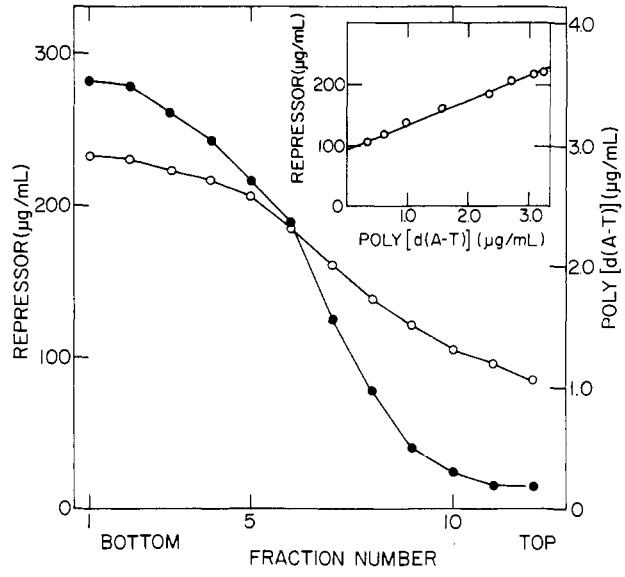

FIGURE 7: Boundary sedimentation velocity experiment with a mixture of repressor and poly[d(A-T)]. Initially, 0.2 mg/mL repressor with 5 base pairs per tetramer poly[d(A-T)] was distributed equally throughout a 5–30% glycerol gradient in the 56 mM ionic strength buffer. The concentrations of repressor (○) and poly[d(A-T)] (●) were determined following centrifugation at 50 000 rpm for 1 h in an SW50.1 rotor at 20 °C. The inset plots [repressor] vs. [poly[d(A-T)]] for fractions 3–10, the boundary region. The slope of this plot yields a stoichiometry for the sedimenting complex of 6.1 base pairs per tetramer.

over a 5-fold range of free repressor concentrations. This indicates that a discrete complex was forming stoichiometrically under all of the conditions employed.

In experiments at 20 mM ionic strength, where 10 μM bis(ANS) was present throughout the gradient, a complex containing 4.9 ± 0.3 base pairs per tetramer was observed. Therefore, this concentration of bis(ANS) does not prevent the stoichiometric binding of repressor to poly[d(A-T)] under these conditions.

Repressor–DNA Binding Monitored by Circular Dichroism. Figure 8A shows CD spectra of a mixture containing four repressor tetramers per 28 base pairs of poly[d(A-T)] and equivalent amounts of repressor and poly[d(A-T)] taken separately. Figure 8B shows CD difference spectra resulting from the interaction of the repressor with poly[d(A-T)], for mixtures containing one, two, or four repressor tetramers per 28 base pairs of DNA. The difference spectrum of two tetramers per 28 base pairs is not simply double that obtained with one tetramer (Figure 8B). This means that the binding of the second tetramer can be distinguished from the first. At both 265 and 270 nm, the change in ellipticity due to one tetramer per 28 base pairs is 60% of that with two tetramers (Figure 8B).

The difference spectrum with four tetramers per 28 base pairs differs from that with two tetramers (Figure 8B). This indicates that more than two repressor tetramers can bind per 28 base pairs of DNA. The ellipticity at 252 nm is noticeably altered by this additional protein, while the ellipticity in the 260–275-nm region remains essentially the same. Clearly, the structural changes caused by this additional protein are quite different than those caused by the first two repressors to bind per 28 base pairs.

Titrations were performed to determine the exact stoichiometries of the different complexes revealed in these difference spectra. A constant amount of repressor was titrated with increasing amounts of poly[d(A-T)] and the resulting ellipticity compared with that of a DNA blank. Figure 9 shows

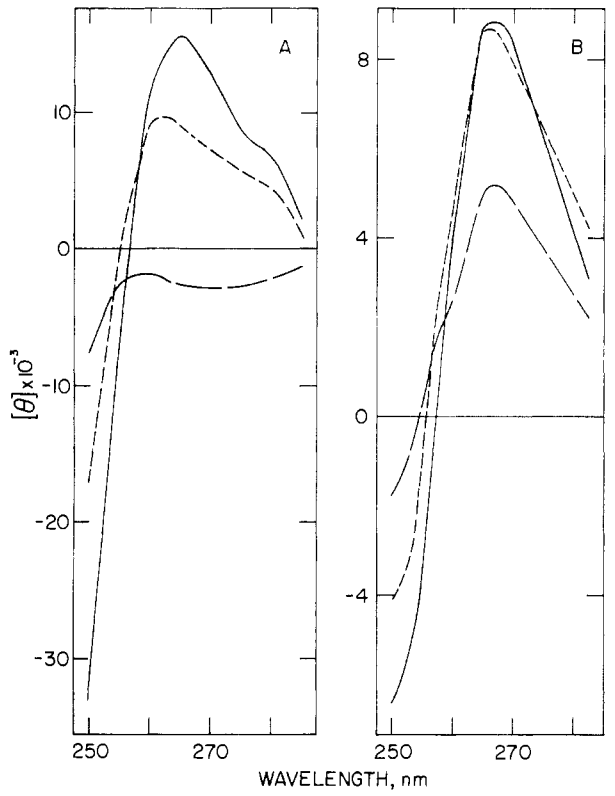

FIGURE 8: CD spectra and difference spectra of repressor bound to poly[d(A-T)]. The observed ellipticities have been adjusted to those of a 1 M nucleotide solution. Corresponding adjustments have been made for the repressor solution. All solutions were prepared with the 11 mM ionic strength buffer. (A) CD spectra of 414 μg/mL repressor mixed with 11 μg/mL poly[d(A-T)], i.e., four tetramers per 28 base pairs (—), 414 μg/mL repressor (– –), and 11 μg/mL poly[d(A-T)] (- - -). A buffer blank was used to establish the base line. (B) CD difference spectra resulting from the interaction of repressor with poly[d(A-T)], with one (– –), two (- - -), or four (—) tetramers per 28 base pairs. These difference spectra were obtained by subtracting the spectra of the repressor and poly[d(A-T)] from the spectrum of the mixture.

titrations in the 11 mM ionic strength buffer, monitored at 265 and 270 nm. These two wavelengths were chosen because they were used by others to monitor repressor–DNA binding (Butler et al., 1977; Durand & Maurizot, 1980). End points were observed at approximately 14 and 28 base pairs per tetramer, i.e., two and one tetramers per 28 base pairs. Above 28 base pairs per tetramer, the plots of the repressor–DNA mixture and the DNA blank have identical slopes, indicating that there were no additional interactions between the protein and DNA. Titrations at 270 nm, carried out at ionic strengths of 30 and 56 mM, yielded the same results as at 11 mM. The average values for these end points from six titrations are 13.3 ± 1.2 and 30.1 ± 4.6 base pairs per tetramer. In a titration monitored at 252 nm in the 11 mM ionic strength buffer (Figure 10), end points were seen at approximately 7 and 14 base pairs per tetramer, i.e., four and two tetramers per 28 base pairs. Titrations at 252 nm, carried out at ionic strengths of 30 and 56 mM, yielded the same results as at 11 mM. The average values for these end points from five titrations are 5.4 ± 0.5 and 16.7 ± 1.7 base pairs per tetramer. These titrations indicate that three distinct complexes are forming, with stoichiometries of approximately one, two, and four tetramers per 28 base pairs.

Manner of Repressor Binding in Repressor–DNA Complexes. Worah et al. (1978) had proposed that the *lac* repressor–nonspecific DNA complexes might contain some protein bound not directly to the DNA but to other repressors.

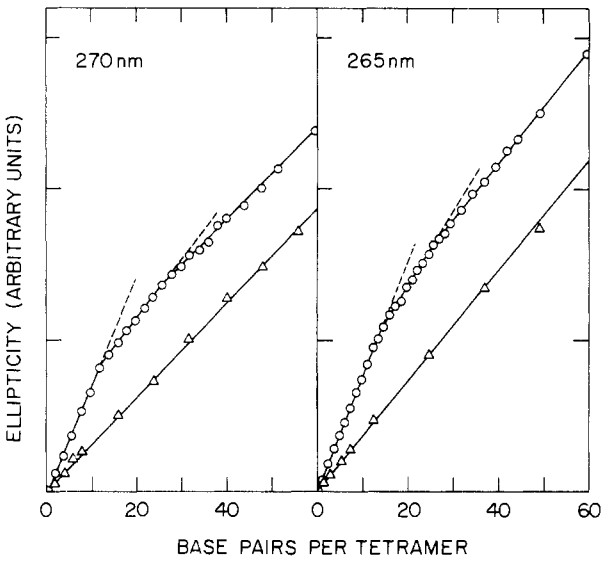

FIGURE 9: CD titrations of repressor with poly[d(A-T)], monitored at 270 and 265 nm. Aliquots of poly[d(A-T)] were added to either the repressor in the 11 mM ionic strength buffer (○) or the buffer alone (△). At 270 nm, the starting repressor concentration was 0.27 mg/mL, and end points were observed at 13 and 27 base pairs per tetramer. At 265 nm, the starting repressor concentration was 0.32 mg/mL, and end points were observed at 15 and 28 base pairs per tetramer.

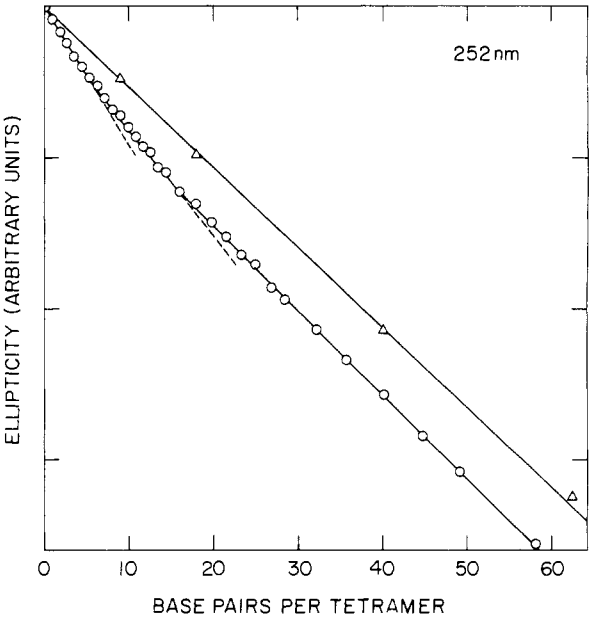

FIGURE 10: CD titration of repressor with poly[d(A-T)], monitored at 252 nm. Aliquots of poly[d(A-T)] were added to either the repressor in the 11 mM ionic strength buffer (○) or the buffer alone (△). The starting repressor concentration was 0.39 mg/mL, and end points were observed at 5.6 and 16 base pairs per tetramer.

Two types of experiments were done in attempts to detect protein–protein associations of this sort. Mixtures containing one repressor tetramer and either one or two repressor cores per 28 base pairs of poly[d(A-T)] were layered on 5–30% glycerol gradients in either the 56 or the 11 mM ionic strength buffer and centrifuged to sediment the repressor–DNA complexes approximately halfway down the tubes. Fractions were then assayed by gel electrophoresis to detect the repressor and core proteins. The sedimenting protein–DNA complex contained only native repressor protein while the repressor cores remained toward the top of the gradient. This experiment demonstrates that the core protein does not bind to repressors bound to DNA. If the *lac* repressor can bind to other re-

pressors already bound to the DNA, then this binding requires the presence of the headpiece regions.

Iodination of tyrosine residues within the repressor's headpiece regions blocks the specific binding of the repressor to its operator (Fanning, 1975). Iodination also inhibits the ability of the repressor to bind to poly[d(A-T)]. Repressor containing 3.3 iodine atoms per subunit was mixed with poly[d(A-T)] to give either 2 or 7 iodinated tetramers per 28 base pairs. When these mixtures were sedimented by using the boundary sedimentation velocity technique, only one repressor tetramer complexed with 28 base pairs of DNA at 56 mM ionic strength. A mixture containing two native and two iodinated tetramers per 28 base pairs of poly[d(A-T)] in 56 mM ionic strength buffer was then centrifuged. While 2 native repressors bound per 28 base pairs of DNA over the sedimentation boundary, essentially no (0.2 tetramer/28 base pairs) iodinated repressor bound.

DISCUSSION

Titrations of the repressor with nonspecific DNA were monitored either by changes in the fluorescence from bis(ANS) bound to the repressor or by changes in the circular dichroism. Both methods revealed that the repressor can form three discrete complexes with nonspecific DNA, with stoichiometries of approximately one, two, and four tetramers per 28 base pairs. The complex containing approximately four tetramers per 28 base pairs also was observed directly in boundary sedimentation velocity experiments.

The repressor possesses at least two different types of bis(ANS) binding sites. The site(s) preferentially populated at low bis(ANS) concentrations have a less polar environment than the majority of sites populated at high concentrations (Figure 3). Apparently, these different sites report the formation of different complexes (Figure 5). The 16 binding sites per tetramer seen in the equilibrium dialysis experiments all have similar affinities for bis(ANS). If, in addition to these sites, each tetramer possessed a single site where bis(ANS) bound more tightly, this could account for the different properties displayed at low and high concentrations of bis(ANS). If the dissociation constant of this single tight binding site were 0.5 μM, then with a total bis(ANS) concentration of 1 μM, twice as many bis(ANS) molecules would have bound to this single tight binding site than to all 16 weak binding sites. At 10 μM bis(ANS), the situation would be reversed, with twice the number of bis(ANS) molecules bound to the weak sites as to the tight site. This shift in where the majority of bis(ANS) was bound could explain the abrupt change in end points going from 1 to 10 μM bis(ANS). A single tight binding site such as this would not have been noticed in the equilibrium dialysis experiments. Other models can be evoked to explain this shift in end points. For example, two or more bis(ANS) molecules bound to the same subunit might behave differently than a single molecule of bis(ANS) and report the formation of a different complex. All four molecules of bis(ANS) which bind per subunit are thought to bind in close proximity to each other, at the interface of the core and headpiece regions (Schneider et al., 1984), and therefore might influence each other's behavior. The frequency of multiple binding would increase dramatically as the concentration of bis(ANS) was increased in the vicinity of its dissociation constant. Whatever the nature of these different binding sites, no one type of site was occupied exclusively during these titrations, causing the fluorescence decrease to be somewhat nonlinear. All of the titrations were carried out with a narrow range of protein concentrations, ensuring that comparable amounts of bis(ANS) were bound to the protein. Consequently, the change in end points occurred over the same range of total bis(ANS) concentrations.

The end point observed with low concentrations of bis(ANS) at ionic strengths $\geq$30 mM is the same as that obtained with ANS using the same ionic strengths (Worah et al., 1978). ANS binds weakly enough to the repressor that only a small number were bound in these experiments, the same as with low concentrations of bis(ANS).

During the titrations monitored with high concentrations of bis(ANS), the addition of 8 base pairs of DNA per tetramer caused the fluorescence to decrease by approximately 60%. Further additions of DNA caused little further decrease in fluorescence. The equilibrium dialysis experiments showed that an excess of DNA displaced approximately 90% of the bis(ANS) bound to the protein (Figure 2). The fact that the fluorescence decrease was less than the amount of bis(ANS) displaced suggests that bis(ANS) molecules bound more tightly fluoresce more. With only 8 base pairs per tetramer, it is likely that only a single subunit on each repressor tetramer directly contacts the DNA (see below), and yet apparently bis(ANS) molecules are displaced from all four subunits. The close packing of protein along the DNA within this complex may contribute to the displacement of bis(ANS).

The boundary sedimentation velocity technique, under conditions where the DNA is saturated with repressor, directly reveals the maximum number of repressor molecules that can complex with a given segment of DNA. This number was approximately five tetramers per 28 base pairs (5.5 base pairs per tetramer), over a range of ionic strengths and free repressor concentrations. While slightly more than the four tetramers per 28 base pairs seen in titrations with high concentrations of bis(ANS), this result clearly shows that at least four tetramers can associate with 28 base pairs of DNA, in the absence as well as the presence of bis(ANS).

Revzin and von Hippel (1977) and O'Gorman et al. (1980) have studied the binding of *lac* repressor to nonspecific DNA using the boundary sedimentation velocity technique. However, the ionic strengths used in these studies, 0.11 M or higher, were too high to allow the formation of the complex containing four repressor tetramers per 28 base pairs of DNA. The binding site size determined by O'Gorman et al. (1980) at 110 mM ionic strength, 11.5 base pairs per tetramer, agrees well with the end points from titrations at 100 mM ionic strength monitored with high concentrations of bis(ANS).

The CD titrations further substantiate the existence of these three complexes. Several conclusions can be drawn from these results. First, these three complexes must form sequentially as DNA is added to the repressor. The fact that three sharp end points are seen demonstrates that, given enough DNA, the complex containing one tetramer per 28 base pairs is favored over that containing two and the complex containing two tetramers is favored over that containing four. In other words, the repressor is held most tightly in the complex containing one, and least tightly in the complex containing four, tetramer(s) per 28 base pairs. If this were not so, then distinct end points corresponding to two and one tetramers per 28 base pairs would not have been seen. Second, the same three complexes are seen at ionic strengths ranging from 11 to 56 mM. This indicates that the two stoichiometries seen at low concentrations of bis(ANS) are due to subtle differences and not to fundamentally different ways of binding. Third, these three complexes form in the absence of bis(ANS) as well as its presence.

These CD studies also provide some information on the nature of these three complexes. The difference spectrum from

260 to 275 nm is contributed primarily by changes in the DNA structure (Butler et al., 1977; Durand & Maurizot, 1980; Maurizot et al., 1974). The first two tetramers to bind per 28 base pairs are almost entirely responsible for generating the difference spectrum in this region, with the first tetramer causing 60% of the maximum change. This suggests that the first two tetramers to be added per 28 base pairs bind directly to the DNA, with the first causing a greater alteration in DNA structure than the second. Since the third and fourth tetramers to bind per 28 base pairs cause no additional change in ellipticity in the 260–275-nm region, these repressors apparently bind with no additional change in DNA structure. Many different features of the double-helical structure of DNA contribute to its ellipticity in the 270-nm region (Johnson et al., 1981), making it difficult to ascribe the observed CD changes to a particular change in DNA structure. Both Butler et al. (1977) and Durand and Maurizot (1980) have discussed possible changes in DNA structure that might account for the observed changes in the CD spectra.

The CD titration data were fit to three straight line segments, giving two end points per titration. These end points agreed with those seen in the fluorescence titrations and with the complex seen in the boundary sedimentation velocity studies. It might be argued that each CD titration could also be fit by two straight line segments connected by a curved region, giving a single, less sharp end point. However, if curvature were present in these plots, it would have increased with ionic strength, because the binding of the repressor to nonspecific DNA is very dependent on ionic strength (Revzin & von Hippel, 1977; deHaseth et al., 1977). The similarity of the data at different ionic strengths strongly argues against the presence of curvature in these plots.

Butler et al. (1977) and Durand and Maurizot (1980) have monitored the association of the *lac* repressor with poly[d(A-T)] using CD and have reported a single end point at 14 base pairs per tetramer. In both studies, it appears that too few data points were recorded to reveal the end point at 28 base pairs per tetramer.

What are the structures of these three complexes? DNase I protection experiments have indicated that the *lac* repressor spans approximately 28 base pairs of the *lac* operator site (Gilbert & Maxam, 1973; Schmitz & Galas, 1979). Electron micrographs have revealed that the repressor spans 25–27 base pairs of nonspecific DNA (Zingsheim et al., 1977). Therefore, it seems likely that the complex containing only one tetramer per 28 base pairs of nonspecific DNA is composed of repressor molecules bound singly to the DNA. This is the strongest mode of binding and leads to the greatest change in DNA structure per repressor. When a second repressor is bound per 28 base pairs, it further perturbs the CD spectrum of the DNA, but not to as great an extent as does the first. This complex is described best by the model proposed by Butler et al. (1977) and Zingsheim et al. (1977), i.e., by repressor molecules bound to opposite sides of the same DNA segment. This double mode of binding is less favorable than the single mode and proceeds to the single mode as more DNA is added. All of the repressors bound doubly might be bound identically, or half might be bound as if singly, and half bound less tightly. Our data cannot distinguish between these alternatives. The third and fourth repressors to bind per 28 base pairs cause little additional change in the DNA structure. If all four tetramers are bound identically, then the extent to which each contacts the DNA is apparently less than for the other two complexes.

One way for four repressor tetramers to bind to 28 base pairs would be for each tetramer to span only 14 base pairs. If repressors then bound to opposite sides of the same DNA segment, this would allow four tetramers to bind per 28 base pairs. Many studies have indicated that a single repressor subunit spans approximately 14 base pairs of DNA. Only two repressor subunits are thought to make strong contacts with the operator during specific binding (Kania & Brown, 1976; Geisler & Weber, 1976; Kania & Muller-Hill, 1977). In NMR studies of isolated headpiece binding to *lac* operator fragments, Nick et al. (1982) have observed a stoichiometry of two headpieces per operator. Scheek et al. (1983) have found that isolated headpieces interact in a specific manner with a 14 base pair fragment comprising the left half of the operator. These results are fully consistent with a model for the repressor–operator complex, derived from X-ray crystallographic studies of CAP, cro, and the λ repressor (Weber et al., 1982; Matthews et al., 1982; Sauer et al., 1982), in which a pair of 2-fold symmetrically oriented headpieces are bound to the two halves of the palindromic operator.

The binding of *lac* repressor to nonspecific DNA is due to ionic interactions (deHaseth et al., 1977; Revzin & von Hippel, 1977) with the headpiece regions (Geisler & Weber, 1977). Schnarr et al. (1983) have studied the binding of isolated headpieces to nonspecific DNA and concluded that two headpieces within the native repressor contact nonspecific DNA. The headpieces are flexibly attached to the core (Buck et al., 1978; Wade-Jardetzky et al., 1979; Jarema et al., 1981). If one of the two headpieces bound to the DNA were to dissociate, this flexibility might allow the repressor to rotate with respect to the remaining headpiece bound to the DNA, such that only one subunit instead of two lay along the DNA. One would now expect a single repressor to span half its normal length of DNA, i.e., 14 instead of 28 base pairs. In fact, Dunaway and Matthews (1980) have shown that a hybrid tetramer containing only a single headpiece binds to nonspecific DNA almost as tightly as tetramers containing two or more headpieces. Whatever the manner in which four repressors bind per 28 base pairs, it appears that all directly contact the DNA. This is indicated by the inability of repressor cores and iodinated repressor to associate with repressor–DNA complexes.

In the complex containing two repressor tetramers per 28 base pairs, the two repressors might be bound identically, or one might be bound more tightly than the other. The second alternative offers a means of explaining why the end point observed in titrations monitored with low concentrations of bis(ANS) changes abruptly going from 25 to 30 mM ionic strength (Figure 6). Perhaps only at low ionic strengths does the second repressor bind tightly enough to DNA to elicit the change in fluorescence. To obtain the full decrease in fluorescence at higher ionic strengths might require enough DNA for all of the repressors to bind singly, i.e., 28 base pairs per tetramer. While the conformation of the second repressor to bind per 28 base pairs may be different at low and high ionic strengths, this second repressor must still cause the same changes in the DNA at both low and high ionic strengths, giving the same CD spectral changes.

The *lac* repressor is known to locate its operator more rapidly than possible by simple diffusion (Barkley, 1981; Winter et al., 1981). It does this by binding to nonspecific DNA at some distance from the operator and then moving along the DNA until it reaches its operator. Winter et al. (1981) have concluded that the repressor slides along the DNA at the rate of one base pair per microsecond, continuously held by ionic interactions. The results presented here suggest that the repressor can bind to nonspecific DNA by a single subunit.

This structure might be an intermediate in the translocation of repressor along DNA. Starting from a position where two subunits were in contact with the DNA, first one subunit would dissociate. The flexible attachment of the headpieces to the core might then allow the repressor to revolve about the remaining headpiece bound to DNA, positioning a second headpiece farther along the DNA. It is conceivable that the repressor might "jump" along the DNA by as much as 30–40 base pairs, i.e., by the length of the repressor. By making one jump of approximately 30 base pairs per millisecond, the repressor would accomplish the same overall motion along the DNA as by sliding one base pair per microsecond, given both motions are random walks.

The relatively low concentration of *lac* repressor inside an *Escherichia coli* cell, 2×10^{-8} M (Gilbert & Muller-Hill, 1966; von Hippel et al., 1974), together with the high ratio of nonspecific DNA to repressor and the high ionic strength, 0.2 M (Kao-Huang et al., 1977), would prevent complexes with two or four tetramers per 28 base pairs from ever forming. However, many biophysical studies are being conducted under conditions where these additional complexes will form. It is important that their presence in these studies be recognized.

Registry No. Poly[d(A-T)], 26966-61-0.

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