Association of *Escherichia coli lac* Repressor with Poly[d(A-T)] Monitored with 8-Anilino-1-naphthalenesulfonate†

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ABSTRACT: The association of *lac* repressor with poly[d(A-T)] was monitored with the fluorescent probe 8-anilino-1-naphthalenesulfonate (Ans). Excess poly[d(A-T)] decreased the emission intensity of the repressor–Ans complex by 30%. Fluorescence titrations indicated that 33 ± 4 base pairs were required to bind all of the repressor. Sedimentation studies indicated, however, that all of the repressor sedimented as a protein–DNA complex with as few as 10 to 15 base pairs per tetramer, even in the presence of Ans. These data are interpreted with two models: one where repressors bind to both sides of the DNA (Butler, A. P., et al. (1977) *Biochemistry 16,* 4757; Zingsheim, H. P., et al. (1977) *J. Mol. Biol. 115,* 565), the other where a double layer of repressors bind to a single side of the DNA. Removal of the amino-terminal regions from the repressor decreased the fluorescence from bound Ans by 77%. The amino-terminal fragments alone did not enhance Ans fluorescence.

The binding of the *lac* repressor protein to its operator site on DNA has served as a model for studying specific protein–DNA interactions (Bourgeois & Pfahl, 1976). The *lac* repressor is capable of recognizing a 17 base pair sequence (Bahl et al., 1977) within the *lac* control region (Gilbert & Maxam, 1973; Dickson et al., 1975) and binding to it with high affinity (Riggs et al., 1970). It also binds to a variety of natural and synthetic DNAs, but with greatly reduced affinities (Lin & Riggs, 1972; Riggs et al., 1972).

The binding to poly[d(A-T)] has been extensively investigated. While the affinity for poly[d(A-T)] is greater than for most nonoperator DNAs, it is still some six orders of magnitude less than for operator DNA (Lin & Riggs, 1972). Binding to poly[d(A-T)] further differs from the specific interaction with operator DNA in being unaltered by inducer binding (Lin & Riggs, 1975; von Hippel et al., 1975). Nevertheless, nonoperator and operator DNA binding share many similarities (Lin & Riggs, 1975). When the amino-terminal 59 amino acid residues are cleaved from the *lac* repressor by tryptic digestion, the ability to bind both operator (Platt et al., 1973) and nonoperator DNA (Lin & Riggs, 1975; von Hippel et al., 1975) is lost; yet the protein retains its tetrameric structure and normal inducer binding (Platt et al., 1973; Files & Weber, 1976). These observations, and many others, strongly implicate the amino-terminal region of the protein in the binding of both operator and nonoperator DNA. Presumably, the major difference between the two is the number and/or type of contacts made between the protein and DNA along the length of their juxtaposition (Kolchinsky et al., 1976).

The structure of DNA is altered by *lac* repressor binding. Wang et al. (1974) have shown that the *lac* operator is unwound approximately 90° when the *lac* repressor binds. Circular dichroism studies indicate that the structure of poly[d(A-T)] is also altered by repressor binding (Maurizot et al., 1974; Butler et al., 1977). Quite likely, the protein simultaneously undergoes conformational changes which serve to strengthen the interaction of the two.

We have used Ans[1] as an environment-sensitive fluorescent probe to look for conformational changes within the *lac* repressor when it binds to poly[d(A-T)]. The binding of Ans to *lac* repressor has been extensively studied (York et al., 1978). This study revealed that Ans, purified by recrystallization of the magnesium salt from water, contains a trace amount of bis(Ans). Due to its much greater affinity for *lac* repressor, this bis(Ans) impurity is responsible for much of the fluorescence increment observed with recrystallized Ans. Pure Ans can be obtained chromatographically. Our present study demonstrates that both recrystallized and column purified Ans can be used to monitor the association of *lac* repressor with poly[d(A-T)]. However, only column purified Ans can be used to study accurately the binding characteristics of Ans in the presence and absence of poly[d(A-T)].

Materials and Methods

lac Repressor Isolation. Escherichia coli CSH 46 (obtained from K. Matthews) which carries i^{SQ} on a heat-inducible prophage, was grown as described by Miller (1972) on a medium described by Betz and Sadler (1976). Typical yields were 30 g wet weight of cells per L, which were stored at −20 °C.

lac repressor protein was purified from 100 g of this frozen cell paste by the method of Rosenberg et al. (1977) with the following modifications. Cell debris pelleted after lysis was washed once with the lysis buffer. The 150-mL phosphocellulose column (2.5 × 30 cm) was eluted with a total gradient volume of 600 mL. Fractions containing *lac* repressor from a 175-mL Sephadex G-200 column (2.5 × 35 cm) were pooled and stored in liquid nitrogen. Typical yields were 80 mg of purified *lac* repressor from 100 g of cell paste.

Purity and Activity of lac Repressor. A single band of protein was visible on $NaDodSO_4$–polyacrylamide gels (Miller, 1972) containing 5 μg of protein. By heavily over-

† From the Department of Chemistry, University of Denver, Denver, Colorado 80208. *Received May 8, 1978.* This work was supported in part by grants from the National Institutes of Health (GM 20561) and the Research Corporation (Cottrell Research Grant). A preliminary report of this work was presented at the 67th Annual Meeting of the American Society of Biological Chemists, San Francisco, California, June 1976.

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[1] Abbreviations used: Ans, 8-anilino-1-naphthalenesulfonate; bis(Ans), bis[4,4′-(8-anilino-1-naphthalenesulfonate)]; EDTA, disodium ethylenediaminetetraacetate; IPTG, isopropyl β-D-thiogalactoside; $NaDodSO_4$, sodium dodecyl sulfate; Tos-PheCH_2Cl, L-1-tosylamido-2-phenylethyl chloromethyl ketone; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/78/0417-4487$01.00/0

loading gels, the protein was shown to be greater than 98% electrophoretically pure. The activity of the *lac* repressor in binding [^{14}C]IPTG (Calatomic 26 mCi/mmol) was measured by equilibrium dialysis (Miller, 1972) under conditions where the *lac* repressor binds a maximum of 2.4 IPTG per tetramer (Ohshima et al., 1974). A Scatchard plot of this data revealed that our protein maximally bound 2.8 IPTG per tetramer. The repressor's specific activity in binding IPTG, expressed as percent excess [^{14}C]IPTG inside the dialysis sac, divided by the protein concentration, was determined as described by Files & Weber (1976) and found to be 1646 % mg^{-1} mL compared with their value of 1676 % mg^{-1} mL for purified *lac* repressor. The operator binding activity of the *lac* repressor was assayed by a filter binding technique (Riggs et al., 1970) using [^{32}P]-λh80dlac(o$^+$)DNA isolated from the doubly lysogenic strain RV/80 (obtained from J. Sadler). This isolation followed the procedure of Betz & Sadler (1976) using 300 μCi of [^{32}P]-phosphoric acid to label 65 mL of culture. These assays were done on protein that had been stored in liquid nitrogen for 4 to 6 months, following all other experiments. The protein was approximately 50% active in binding *lac* operator.

Lac Repressor Cores and Headpieces. Repressor cores were prepared by the method of Files & Weber (1976): 11.6 mg of repressor was digested by 1% (w/w) Tos-PheCH$_2$Cl-trypsin (Worthington) for 10 min at 25 °C. Fractions from a 1 × 25 cm Sephadex G-50 column containing repressor cores were pooled and precipitated with $(NH_4)_2SO_4$ (243 mg/mL). This precipitate was redissolved and stored at −20 °C in 1.1 M Tris-HCl (pH 7.6 at 4 °C), 0.3 mM dithiothreitol, 30% (v/v) glycerol. NaDodSO$_4$-polyacrylamide gels (Miller, 1972) revealed that 73% of this core preparation was band A, i.e., missing only the amino-terminal 59 amino acid residues. The remainder was found as bands B (17%) and C (10%) where some carboxyl-terminal residues were also removed. These cores retained full inducer binding activity with a specific activity of 1772 % mg^{-1} mL in the assay previously described.

Repressor headpieces were prepared by the method of Geisler & Weber (1977): 5–7 mg of repressor was digested with Tos-PheCH$_2$Cl-trypsin (Worthington) as described and applied to a 1.6 × 95 cm Sephadex G-150 column equilibrated with 0.1 M NH_4HCO_3. Repressor headpieces emerged from this column before the glycerol contained in the digestion buffer. Fractions containing headpieces were pooled and concentrated by one of three methods: lyophilization, precipitation with $(NH_4)_2SO_4$ (560 mg/mL), or adsorption onto phosphocellulose (Whatman P-11) followed by elution with 1 M potassium phosphate, pH 7.5. Headpieces concentrated by the latter two methods were then run through a Sephadex G-25 column equilibrated with a standard buffer described below. These headpiece preparations were judged to be greater than 85% pure using NaDodSO$_4$-polyacrylamide gels made with 20% acrylamide.

Other Materials. Ans was purified according to York et al. (1978). Poly[d(A-T)], with s_{20} values ranging from 8.5 to 13.0, was obtained from Miles Laboratories. Stock solutions containing approximately 4 mg/mL poly[d(A-T)] were stored at 4 °C in a standard buffer used for most experiments. This buffer contained 0.1 M Tris-HCl, pH 8.0, at 25 °C, and 0.3 mM dithiothreitol. The T_m of poly[d(A-T)] in standard buffer is 57 °C. All other chemicals were reagent grade.

Preparation of Experimental Solutions. Just prior to use, stock solutions of *lac* repressor and repressor cores were thawed, diluted if necessary to less than 3 mg/mL with the buffer being used in the experiment, and dialyzed for 3 to 4 h against this buffer at 4 °C, with changes every hour. The concentrations of most components were determined spectrophotometrically using the following values. *lac* repressor: $A_{280nm}^{1mg/mL} = 0.59$ (Huston et al., 1974), subunit mol wt = 37 500 (Beyreuther et al., 1973). *lac* repressor cores: $A_{280nm}^{1mg/mL} = 0.57$ (Huston et al., 1974), subunit mol wt = 30 000 (Files & Weber, 1976). Ans: $\epsilon_{351nm} = 6000$ M^{-1} cm^{-1} (Ferguson & Cahnmann, 1975). Poly[d(A-T)]: $A_{260nm}^{1mg/mL} = 20$ (Lin & Riggs, 1972). *lac* repressor headpiece concentrations were determined by the method of Lowry et al. (1951) using bovine albumin, fraction V (Sigma) as a standard. The concentration determined in this manner was essentially identical with that determined by absorbance using $A_{280nm}^{1mg/mL} = 0.88$, calculated from the amino acid composition by the method of Wetlaufer (1962). The molecular weight of headpieces was assumed to be 6000 (Geisler & Weber, 1977).

Fluorescence Measurements. All fluorescence measurements were performed on a Perkin-Elmer MPF-3 fluorescence spectrophotometer, using a 4-mm pathlength cuvette thermostated to 25 °C. Except where noted, fluorescence intensities were corrected for contributions made by unbound Ans, repressor, buffer, dilution effects, and inner filter effects. The details of these procedures are contained in York et al. (1978).

Sedimentation through Glycerol Gradients. Sample (0.20 mL) in standard buffer was layered on 5.1 mL of standard buffer containing a 5–30% (v/v) glycerol (MCB Spectroquality) gradient. The amounts of protein and poly[d(A-T)] placed on the gradient were determined spectrophotometrically. These gradients were centrifuged 2 h at 50 000 rpm in a Spinco SW50.1 rotor at 23 °C. Fractions (0.41 mL) were assayed for A_{260nm} and for protein by the method of Lowry et al. (1951) following precipitation by 8% trichloroacetic acid at 0 °C. Protein concentrations determined by this method were then corrected to give values consistent with those determined spectrophotometrically. In experiments where Ans was included, both the sample and gradient contained 5.0 × 10^{-5} M column purified Ans.

Results

Poly[d(A-T)] Binding to the lac Repressor Detected by Ans Fluorescence. Ans binds to the *lac* repressor enhancing its fluorescence intensity and blue shifting its emission maximum (York et al., 1978). The emission spectrum of the column purified Ans–repressor complex is altered by the addition of poly[d(A-T)] DNA (Figure 1). When 54 base pairs of poly[d(A-T)] are added per repressor tetramer, the intensity at the emission maximum is decreased 25% without significantly altering the wavelength of maximum emission. Essentially the same decrease is observed using recrystallized Ans in place of column purified Ans. In contrast, the emission spectrum of Ans alone is not altered by the addition of poly[d(A-T)].

This decrease in fluorescence intensity can be followed as poly[d(A-T)] is progressively added to a mixture of repressor and Ans. Figure 2 shows one such titration where, except for an increase in intensity caused by the first addition of poly[d(A-T)] which will be discussed later, the emission intensity decreases linearly until a sharp break is observed after 29 base pairs of poly[d(A-T)] have been added per repressor tetramer. From there on, the fluorescence intensity decreases very slowly. The two straight line segments in this plot were obtained by a least-squares analysis, omitting points close to the intersection of the two lines.

This experiment has been repeated under a variety of conditions: using several different samples of poly[d(A-T)]; with *lac* repressor prepared and stored by the method of Platt et al. (1973) as well as by the method of Rosenberg et al. (1977);

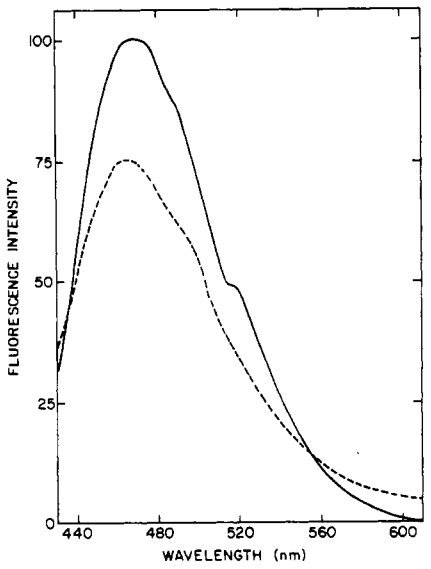

FIGURE 1: Fluorescence emission spectra of the column purified Ans-repressor complex in the absence (—) and presence (- - -) of poly[d(A-T)]. Excitation wavelength = 350 nm. All solutions contained standard buffer. [Ans] = 4.7×10^{-5} M; [repressor subunits] = 1.2×10^{-5} M; [poly[d(A-T)]] = 0.10 mg/mL (54 base pairs/tetramer) when present.

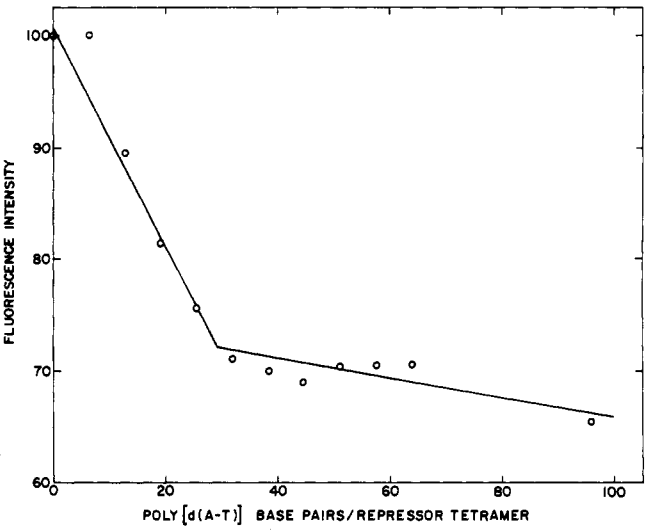

FIGURE 2: Titration of recrystallized Ans-repressor complex with poly[d(A-T)]. Excitation and emission wavelengths, 350 and 470 nm, respectively. Starting solution: 450 μL, containing [Ans] = 8.2×10^{-5} M, [repressor subunits] = 1.1×10^{-5} M, 0.3 mM magnesium acetate, 0.1 mM EDTA, in standard buffer. One-microliter additions of 4.8 mg/mL poly[d(A-T)] each gave 6.4 base pairs/tetramer. The fluorescence intensity was obtained by subtracting contributions of Ans, poly[d(A-T)], and buffer from the total observed fluorescence, correcting for inner filter and dilution effects. The repressor alone contributes approximately 4% of the initial fluorescence.

with recrystallized or column purified Ans; in the presence or absence of dithiothreitol (0.3 mM), magnesium acetate (0.3 mM), and EDTA (0.1 or 0.3 mM). All of these experiments gave very similar results; the fluorescence of the repressor-Ans complex decreased linearly as poly[d(A-T)] was added until a sharp end point was reached, leaving approximately 70% of the original fluorescence increment that either remained stable or decreased very slowly as more poly[d(A-T)] was added. A least-squares analysis of eight such titrations yields an average end point of 33 ± 4 base pairs of poly[d(A-T)] per repressor tetramer.

The poly[d(A-T)] used in these experiments had an average molecular weight exceeding 5×10^5. Thus, the average DNA molecule contained more than 25 tetramer binding sites. The size of the repressor binding site determined in these experiments therefore should not be significantly influenced by having less than a full binding site at the end of a DNA molecule.

When only a small amount of poly[d(A-T)] is added to the *lac* repressor, generally less than 5 to 10 base pairs per tetramer, a precipitate usually forms. This precipitate disappears as the number of base pairs per tetramer is increased to ten or more. In the titration shown in Figure 2, the anomolously high fluorescence after the first addition of poly[d(A-T)] is correlated with the formation of a precipitate and consequently increased light scattering.

It was often difficult to analyze titrations during which precipitation had occurred as two linear segments. For this reason, six of the eight titrations analyzed to yield the size of the repressor binding site were those in which there was no precipitation. The many titrations which underwent precipitation and were not included in this analysis did, nevertheless, display clearly defined endpoints. The size of the repressor binding site determined from these experiments agreed well with that reported.

Lac Repressor Has a Low Affinity for Ans. The decreased emission of the Ans-repressor complex when poly[d(A-T)] is added could be caused by a decrease in (1) the number of binding sites for Ans, (2) the affinity for Ans, allowing some of the bound Ans to dissociate, or (3) the quantum yield of bound Ans. In order to distinguish between these alternatives, we have endeavored to measure the dissociation constant(s) and number of binding sites for Ans in the absence and presence of poly[d(A-T)]. These parameters were evaluated by carrying out two titrations, one where the concentration of Ans was varied while the concentration of repressor subunits was fixed at 1.3×10^{-5} M, and one where [repressor subunits] was varied while [Ans] was fixed at 1.3×10^{-5} M (York et al., 1978). Column purified Ans was used for these experiments to avoid the complications introduced by the bis(Ans) impurity in recrystallized Ans.

With poly[d(A-T)] absent, these two titrations yielded coincident linear plots (Figure 3). Similar results were obtained in the presence of excess poly[d(A-T)] (60 base pairs per tetramer), except that the slope of the plots was 75% of that obtained in the absence of poly[d(A-T)]. The coincidence together with the linearity of these plots demonstrates that the values of K_{DA} and K_{DA}/n_A, where K_{DA} is the dissociation constant for Ans and n_A is the number of Ans binding sites per subunit, are too large to evaluate, both in the absence and presence of poly[d(A-T)]. The limited solubility of *lac* repressor in standard buffer (3 mg/mL) and inner filter effects from Ans prevent us from extending these titrations to higher concentrations. Therefore, it has not been possible to determine why the fluorescence from bound Ans decreases when *lac* repressor binds to poly[d(A-T)].

Sedimentation of Repressor-Poly[d(A-T)] Complexes. The binding of *lac* repressor to poly[d(A-T)] was also followed by sedimenting mixtures of the two through glycerol gradients. These experiments yielded information concerning (1) the ability of the *lac* repressor to complex with poly[d(A-T)], (2) the number of base pairs of poly[d(A-T)] required to complex the repressor, and (3) the effect of Ans on the association of repressor with poly[d(A-T)].

Figure 4 shows the results of a sedimentation experiment carried out in the absence of Ans. Repressor alone was found as a peak in fractions 9 through 11 (Figure 4A), accounting

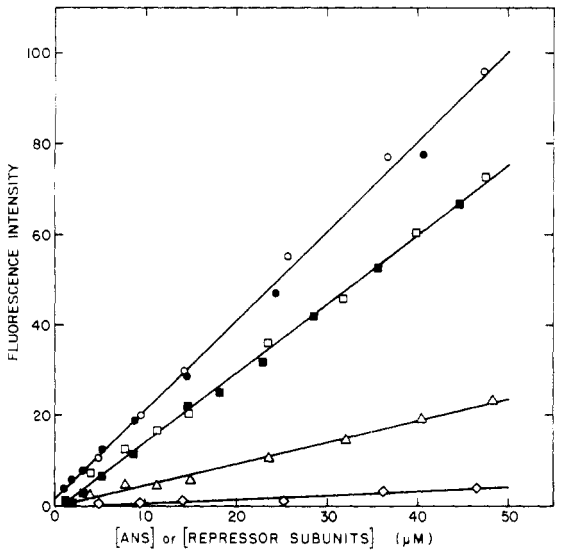

FIGURE 3: Titrations of *lac* repressor (○ and ●), *lac* repressor complexed with 60 base pairs of poly[d(A-T)] per tetramer (□ and ■), repressor cores (△), and repressor headpieces (◇) with column purified Ans. Open symbols: [protein monomers] held constant at 1.3×10^{-5} M and [Ans] varied. Solid symbols: [Ans] held constant at 1.3×10^{-5} M and [repressor subunits] varied. All solutions contained standard buffer. Excitation and emission wavelengths, 350 and 470 nm, respectively. Least-squares fits of the data to straight lines gave the following correlation coefficients: (○ and ●) 0.998; (□ and ■) 0.999; (△) 0.995; (◇) 0.946.

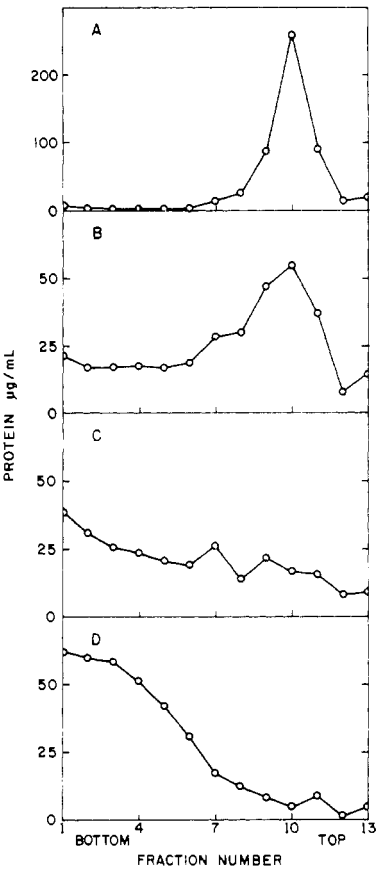

FIGURE 4: Sedimentation of *lac* repressor and *lac* repressor complexed with poly[d(A-T)] through glycerol gradients. The samples contained: (A) 199 μg of repressor; (B) 216 μg of repressor and 5.9 μg of poly[d(A-T)] (7 base pairs/tetramer); (C) 213 μg of repressor and 13.5 μg of poly[d(A-T)] (15 base pairs/tetramer); and (D) 208 μg of repressor and 26.4 μg of poly[d(A-T)] (31 base pairs/tetramer). The poly[d(A-T)] had an s_{20} value = 9.5, determined by Miles Laboratories.

for 88% of the input protein (98% of the input protein was recovered in fractions 7 through 13). Under identical conditions, poly[d(A-T)] alone formed a peak in fractions 7 through 10 (data not shown). Figures 4B through 4D show the results obtained with 7, 15, and 31 base pairs per tetramer of poly[d(A-T)] present. The amount of protein remaining in fractions 9 through 11 provides an estimate of the amount of repressor not complexed with poly[d(A-T)]. For Figures 4B through 4D, this amount was 25, 9, and 4%, respectively, of the input protein. Due to pelleting of the repressor–poly[d(A-T)] complex, not all of the input protein was recovered from these gradients. However, in other experiments where the repressor–poly[d(A-T)] complex was not pelleted, recovery of the input protein was quantitative.

The data shown in Figure 4D indicate that the repressor protein used for these experiments was at least 95% active in complexing with poly[d(A-T)]. With 61 base pairs per tetramer of poly[d(A-T)] present, less than 1% of the input protein was found in fractions 9 through 11 (data not shown). The data shown in Figures 4B and 4C indicate that 10 to 15 base pairs per tetramer of poly[d(A-T)] are required to fully complex the repressor. These experiments were repeated with 5.0×10^{-5} M column purified Ans in both the gradient and sample. The presence of Ans had no effect on the sedimentation behavior of these mixtures of repressor and poly[d(A-T)].

Removal of the Amino-Terminal Region from the lac Repressor Greatly Affects Ans Binding. Repressor cores, lacking the amino-terminal 59 amino acid residues, were prepared as described in Materials and Methods. While retaining full inducer binding activity, these repressor cores had lost the ability to bind to poly[d(A-T)]. When a mixture of repressor cores and excess poly[d(A-T)] in standard buffer was sedimented through a glycerol gradient, both the cores and poly[d(A-T)] sedimented as they did separately. These repressor cores also bound column purified Ans differently than native repressor. A titration of 1.3×10^{-5} M repressor cores with column purified Ans again gave a linear increase in fluorescence intensity. However, the magnitude of this increase was only 23% of that observed with native repressor (Figure 3).

A comparison was also made of the abilities of repressor headpieces, i.e., the amino-terminal fragments, and native repressor to enhance the fluorescence of Ans. A titration of 1.3×10^{-5} M headpieces, concentrated by $(NH_4)_2SO_4$ precipitation, with column purified Ans gave a linear increase in fluorescence intensity only 4% of that observed with native repressor (Figure 3). Similar results were obtained with headpieces concentrated by either lyophilization or adsorption onto phosphocellulose. A mixture of repressor cores and headpieces concentrated by lyophilization gave only the fluorescence increase obtained with repressor cores alone.

Discussion

The association of *lac* repressor with poly[d(A-T)] can be monitored by the decrease in fluorescence intensity from repressor-bound Ans. This decrease is linear, beginning with the fluorescence intensity of the Ans–repressor complex and concluding with a sharp end point, when approximately 70% of the original fluorescence increment remains. The linearity of this decrease suggests that a single process is affecting the fluorescence of Ans. The sharp end point indicates that the binding of repressor to poly[d(A-T)] is stoichiometric under the experimental conditions employed, as expected from the results of Lin & Riggs (1972) and the cosedimentation of repressor with poly[d(A-T)] such as shown in Figure 4D.

These fluorescence titration experiments imply that each

repressor tetramer shields an average of 33 ± 4 base pairs of poly[d(A-T)] from other repressor tetramers.[2] In contrast, our sedimentation studies indicate that 10 to 15 base pairs of poly[d(A-T)] are sufficient to fully complex the repressor in a rapidly sedimenting protein-DNA complex. Furthermore, the presence of Ans does not alter this behavior. It appears from these results that repressor binds to poly[d(A-T)] in fundamentally different ways with 15 and 30 base pairs per tetramer.

Butler et al. (1977) followed the association of *lac* repressor with nonoperator DNA by monitoring changes within the circular dichroism spectrum of the DNA. They concluded that 13 base pairs per tetramer were sufficient to bind the repressor. This data and the electron microscopic data of Zingsheim et al. (1977) have led both to propose that a single repressor tetramer covers 26 base pairs of DNA, but that the apparent site size is 13 base pairs because repressor binds to both sides of nonoperator DNA. To explain our fluorescence titration data with this model requires a somewhat unusual set of circumstances. For the fluorescence decrease to remain linear up to 30 base pairs per tetramer, repressors bound singly along the DNA would have to experience twice the decrease in fluorescence intensity as repressors bound doubly along both sides of the DNA. Furthermore, given 30 base pairs per tetramer, all of the repressor would have to be bound in a single layer along the DNA. These difficulties in reconciling our data with this model prompt us to tentatively offer an alternative model.

This model states that only the direct binding of repressor with poly[d(A-T)] decreases the fluorescence intensity of bound Ans, i.e., that approximately 30 base pairs per tetramer are required for all of the protein to bind directly to DNA. To account for our sedimentation results, this model proposes that the *lac* repressor is capable of binding to other repressor molecules that have already saturated the poly[d(A-T)]. Accordingly, with 15 base pairs per tetramer, a double layer of tetramers would be bound to a *single* side of the DNA. This model is suggested by the tendency of the *lac* repressor to aggregate with itself to the point where it precipitates (Laiken et al., 1972; Maurizot et al., 1974). The association of protein with protein bound to DNA might be made even more specific by conformational changes which occur in the *lac* repressor as a consequence of its association with poly[d(A-T)] (unpublished experiments).

This model is reasonably consistent with most of the existing data concerning nonoperator DNA. Revzin & von Hippel (1977), on the basis of extensive sedimentation velocity experiments, concluded that 10 to 14 base pairs per tetramer of nonoperator DNA were sufficient to incorporate all of the repressor into a high molecular weight protein-DNA complex. Our sedimentation studies using glycerol gradients reaffirm these results. But again, the size of the repressor binding site on DNA may be underestimated in these studies due to protein-protein associations. Zingsheim et al. (1977) have recently presented electron microscopic evidence that a double layer of repressor tetramers binds to nonoperator DNA. Their interpretation was that *lac* repressor binds to both sides of the DNA helix, but they acknowledge that pairs of repressor tetramers could be binding to one side of the DNA. Unfortunately, the position of the DNA in these complexes could not be delineated. Finally, Wang et al. (1977) have observed the effect of different amounts of repressor on the thermal melting of poly[d(A-T)]. While the maximum alteration in melting behavior was obtained with 13 base pairs per tetramer, a rather distinct break in melting behavior was also noted between 26 and 33 base pairs per tetramer. In fact, with 26 base pairs per tetramer, the melting behavior of all the DNA was altered.

The only result that is difficult to reconcile with this model comes from the circular dichroism studies of Butler et al. (1977). This model would require a double layer of tetramers bound to a single side of the DNA to induce twice the change in the circular dichroism spectrum of the DNA as a single layer. Certainly, more experiments are needed to fully describe the stoichiometry of repressor-poly[d(A-T)] association.

The precipitation which we frequently observe with small amounts of poly[d(A-T)], i.e., with less than 10 base pairs per tetramer, presumably results from the aggregation of large protein-DNA complexes, for under our experimental conditions repressor alone is quite soluble at these concentrations. This aggregation process appears to be promoted by free repressor, for it does not occur in the absence of free repressor. This phenomenon might account for the observation of Maurizot et al. (1974) that the light scattering from a solution of poly[d(A-T)], to which repressor was progressively added, sharply increased when the ratio of base pairs per tetramer decreased below 10 to 15.

The amino-terminal 59 residues can be selectively cleaved from the *lac* repressor by limited tryptic digestion, leaving a tetrameric core protein that binds inducer normally but not operator (Platt et al., 1973; Files & Weber, 1976). The conditions of digestion can be modified to yield the amino-terminal region intact, as a mixture of peptides encompassing residues 1 to 59 and 1 to 51, which are referred to as headpieces (Geisler & Weber, 1977). These headpieces retain some affinity for DNA.

We have investigated the binding of Ans to repressor cores and headpieces in order to locate the Ans binding sites on the repressor protein. The repressor core protein enhances the fluorescence of Ans much less than does native repressor, indicating that Ans binding sites on the native repressor have been modified or lost by removing the amino-terminal regions. Since repressor headpieces show little ability to enhance the fluorescence of Ans, it seems plausible that these Ans binding sites are formed through the interaction of the core and headpiece portions of the native repressor structure, quite likely in a region where these two portions make contact. The binding of poly[d(A-T)], presumably to the headpiece portions, might then alter this contact region in a way which either displaces the bound Ans or changes its quantum yield.

The low affinity of *lac* repressor for Ans has prevented us from determining whether poly[d(A-T)] directly competes for Ans binding sites on the protein, or induces a conformational change in the protein which alters the properties of its Ans binding sites. The fact that the fluorescence of the Ans-repressor complex is altered by poly[d(A-T)] binding does encourage a study of other noncovalently held fluorescent probes that are bound more tightly. Certainly, bis(Ans) is a most attractive probe in this respect.

[2] The size of the repressor binding site reported here has not been corrected for incomplete saturation of the poly[d(A-T)] (McGhee & von Hippel, 1974; Butler et al., 1977). This correction should be small, however, for the sharp break obtained in our fluorescence titrations indicates that the binding of repressor to poly[d(A-T)] is essentially stoichiometric. Even with only 80% saturation of the poly[d(A-T)], the size of the tetramer binding site would still be 26 base pairs.

Acknowledgments

The authors thank Dr. T. C. Rounds for his critical reading of this manuscript and computer analysis of some of the data, Dr. J. R. Sadler for the RV/80 strain and many detailed laboratory protocols, Dr. K. S. Matthews for the CSH46 strain,

and Mr. D. E. Kelsey for his assistance in preparing *lac* repressor and assaying its activity.

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