DNA Sequence Dependent and Independent Conformational Changes in Multipartite Operator Recognition by λ-Repressor†

Sunanda Deb, Sumita Bandyopadhyay, and Siddhartha Roy*

Department of Biophysics, Bose Institute, P-1/12 CIT Scheme VII M, Calcutta 700 054, India

Received August 25, 1999; Revised Manuscript Received December 8, 1999

ABSTRACT: Binding of regulatory proteins to multipartite DNA binding sites often occurs with protein−protein interaction, resulting in cooperative binding. The operators of bacteriophage λ have several pairs of repressor binding sites (O_R1-O_R2, O_R2-O_R3, O_L1-O_L2, and O_L2-O_L3) separated by a variable number of base pairs, and thus, bacteriophage λ is a model system for studying multipartite operator recognition by DNA-binding proteins. Near-UV circular dichroism spectra show that the DNA is distorted in O_R1-O_R2 and O_L2-O_L3 but much less so in O_R2-O_R3. Upon titration of λ-repressor with single-operator sites O_R1, O_R2, and O_R3, it was observed that the tryptophan fluorescence quenches to different degrees, suggesting different conformations of the protein in the three DNA−protein complexes. Acrylamide quenching of tryptophan fluorescence of λ-repressor bound to these single operators also shows different Stern−Volmer constants, supporting the above conclusions. Titration of λ-repressor with oligonucleotides containing pairs of operator sites also causes different degrees of fluorescence quenching. In particular, fluorescence quenching induced by O_R1-O_R2 binding is less than the quenching induced by either of the single operators alone, suggesting additional conformational changes upon establishment of protein−protein contact. Stern−Volmer constants obtained from acrylamide quenching of tryptophan fluorescence of λ-repressor bound cooperatively to pairs of operator sites are different from those of the single-operator-site-bound repressors. For example, O_R2-O_R3-bound repressor has significantly higher acrylamide quenchable components than either of the O_R2- or O_R3-bound proteins, again suggesting additional conformational changes upon establishment of protein−protein contact. We conclude that the strategy of recognition of multipartite operator by λ-repressor is complex and varied, involving conformational changes in both DNA and protein that are determined by the separation of the binding sites as well as the nucleic acid sequence.

Recognition of specific nucleic acid sequences by proteins is at the heart of gene expression and its regulation. Despite advances in the understanding of the structural and molecular basis of specificity, much remains unexplored. DNA-binding proteins often bind as dimers to DNA sequences having pseudodyad symmetry. Cocrystal structures of several repressors with their cognate operators have demonstrated the importance of protein side-chain hydrogen bonding with that of the base atoms in the major or minor groove of DNA (*1*−*6*). However, the role of the conformational changes of the operator DNA or of the protein in the recognition process is not well understood. As has been seen in many cocrystals, the operator is distorted in the complex, suggesting that the distortion plays a crucial role in the recognition process. In addition, there is now increasing evidence that the conformation of the regulatory protein is changed upon binding to the cognate DNA sequence, which may profoundly influence the nature of the DNA−protein interaction (*7*, *8*).

Many DNA binding sites exist in multipartite form (*9*). Recognition of multipartite binding sites by DNA-binding proteins involves binding of dimers to two or more such sites with cooperativity (*10*). Cooperative binding of regulatory proteins to these multipartite operators occurs with protein−protein interaction (*11*−*14*). Classical examples of multipartite operators are the right and left operators of bacteriophage λ. The right and left operators of bacteriophage λ have three operator sites each, O_R1, O_R2, and O_R3 and O_L1, O_L2, and O_L3, respectively. Each operator site is capable of binding a repressor dimer (*15*). The binding of repressor dimers to adjacent sites is accompanied by protein−protein contact between two repressor dimers. Different numbers of base pairs separate the operator sites. For example while the sites in the O_R1-O_R2 and O_L1-O_L2 pairs are separated by six base pairs, the sites in the O_R2-O_R3 pair are separated by seven base pairs, and the sites in the O_L2-O_L3 pair are separated by three base pairs (*15*). Disregarding the possible microheterogeneity in DNA structure, the center to center distances between the operator sites may vary from 69 to 83 Å and the orientations may vary from −72° to +72°. Clearly flexibility of the λ-repressor or that of the DNA would be crucial for recognition of such different pairs of operator sites.

Quantitative footprint titrations have provided estimates of binding and interaction energies (*16*). Surprisingly, the net cooperative interaction energies are very similar in all cases, amounting to 2−3 kcal/mol. Despite such significant

† We acknowledge financial support from the Council of Scientific and Industrial Research (India).

* To whom correspondence should be addressed. Fax: 91-33-334-3886. E-mail: sidroy@vsnl.com.

10.1021/bi9919955 CCC: $19.00
Published on Web 03/04/2000

differences in distances and orientations, how the recognition of different pairs of operator sites is achieved with similar net interaction energies remains to be explored. We address this issue in this paper using circular dichroism and fluorescence spectroscopy.

MATERIALS AND METHODS

Materials. Crude O_R1 (5′ AACCATTATCACCGCCAGAGGTAAAATAG 3′ and its complementary oligonucleotide), O_R2 oligonucleotide (5′ AAC CAT TAA CAC CGT GCG TGT TGA AAT AG 3′ and its complementary sequence), O_R3 oligonucleotide (5′ AAC CAT TAT CAC CGC AAG GGA TAA AAT AG 3′ and its complementary sequence), O_R1-O_R2 oligonucleotide (5′ CCT ATC ACC GCC AGA GGT AAA ATA GTC AAC ACG CAC GGT GTT ACC A 3′ and its complementary sequence), O_R2-O_R3 oligonucleotide (5′ CTA TCA CCG CAA GGG ATA AAT ATC TAA CAC CGT GCG TGT TGC 3′ and its complementary sequence), and O_L2-O_L3 oligonucleotide (5′ GAT AAC CAT CTG CGG TGA TAA ATT ATC TCT GGC GGT GTT GAC 3′ and its complementary sequence) were purchased from IMMCO GENETICS Private Limited (New Delhi, India). QAE-Sephadex A-50 was purchased from Pharmacia United (Sweden). Ampicillin, IPTG, and PMSF were purchased from Sigma Chemical Co. (St. Louis, MO). Three times recrystallized acrylamide used for quenching studies was purchased from Spectrochem (India). Bactotryptone and Yeast Extract were purchased from Difco Laboratories (Detroit, MI). Bis-ANS[1] was purchased from Molecular Probes Inc. (Eugene, OR). All other reagents were of analytical grade.

Purification of λ-Repressor. The repressor was purified according to ref *17*. Y210C repressor was purified according to ref *18*.

Purification of Oligonucleotides. All the oligonucleotides were purified by reversed phase high-performance liquid chromatography (HPLC) (μ-Bondapack C18 column). The oligonucleotides mixed at 1:1 molar ratio were annealed in 0.1 M potassium phosphate buffer, pH 8, by heating at 80 °C and then cooling slowly to room temperature.

Circular Dichroism. Circular dichroism (CD) spectra were measured in a JASCO J-600 spectropolarimeter using a path length of 1.0 cm for the near-UV measurements. The measurements were performed in 0.1 M potassium phosphate buffer, pH 8.0, at ambient temperature (25 ± 1 °C). The near-UV CD spectra were measured as described by Bandyopadhyay et al. (*8*).

Fluorescence Methods. All fluorescence spectra were measured in a Hitachi F 3010 spectrofluorometer having a facility for spectra addition and subtraction. The excitation and emission band-passes were 5 nm, unless mentioned otherwise. Fluorescence quenching studies were done as described in ref *19*. Operator-induced conformational change was measured by titrating 1 μM repressor with duplex oligonucleotides containing the appropriate sequence of the operator site(s), in 0.1 M phosphate buffer, pH 8.0. Acrylamide quenching studies were done at 1 μM protein concentration in 0.1 M potassium phosphate buffer, pH 8.0, with 0.5 μM single-operator sites or 0.25 μM double-operator sites. The single-site dissociation constants of all the operator sites are significantly lower than the protein concentration used here, and hence all operator sites will largely be saturated under the experimental conditions used. The differences in occupancy in different cases are small. Use of higher protein concentrations may involve the possibility of noncooperative tetramer formation and was thus avoided. The correction due to insufficient occupancy will be of any quantitative significance only in the case of the weakest single operator O_R3. Even in such a case, we estimate that there will be no significant change in the qualitative pattern or conclusions drawn. The observed fluorescence was corrected for the inner filter effect as described (*19*). The excitation wavelength was 295 nm, and the emission was measured at 340 nm. Bis-ANS binding to free wild-type repressor and that to double-operator-site complexes were carried out on the same day to avoid day to day variations. Repressor (1 μM) or its complex with 0.25 μM oligonucleotides was titrated with increasing concentrations of bis-ANS. The excitation wavelength was 455 nm, and the emission wavelength was 500 nm.

Data Analysis. All experiments were repeated between three and six times. The error bars shown in the figures are based on such repetitions. The quenching data were fitted to the Stern−Volmer equation having two or three quenchable components using Sigma Plot (SPSS Inc.). The standard errors of the Stern−Volmer constant and quenchable fractions reported in the table and the text are based on three or more independent experiments and corresponding fitted parameters.

RESULTS

There are now increasing instances where a single DNA-binding protein binds to multiple binding sites with cooperativity, involving protein−protein interaction (*9*). In some cases these sites are adjacent, as in the right and left operators of bacteriophage λ (*15*). They may also be far apart as in *lac* (*20*−*22*) and *gal* operons of *E.coli* (*12*, *23*). The distance and the relative orientation of these sites vary, even when they bind the same protein. Whether such variable separations have a biological significance or are merely a result of overall structural and functional constraints imposed upon that region of the DNA sequence is not known. From the point of view of molecular recognition, these systems are most interesting. For example, in the λ-repressor/operator system in the natural context, binding of two operator sites cooperatively by the tetrameric species is the relevant reaction. The interactions that characterize these complexes have to be elucidated. The first step toward such an understanding is to observe the conformation of the DNA sequence that separates the two operator sites.

One of the best ways to study DNA distortion is by CD spectroscopy. CD spectra of double-stranded DNA are dominated by nearest-neighbor base interactions, which in turn are sensitive to the distance and orientation of the neighboring bases (*24*). We have previously shown that it is possible to measure DNA distortion in an oligonucleotide containing the two operator sites O_R1 and O_R2 by difference CD spectroscopy (*8*). Figure 1 shows the CD spectra of oligonucleotides containing O_R1-O_R2, O_R2-O_R3, and O_L2-

[1] Abbreviations: Bis-ANS, 1,1′-bi(4-anilino)naphthalene-5,5′-disulfonic acid; CD, circular dichroism; IPTG, isopropyl-β-thiogalactoside; PMSF, phenylmethyl sulfonyl fluoride.

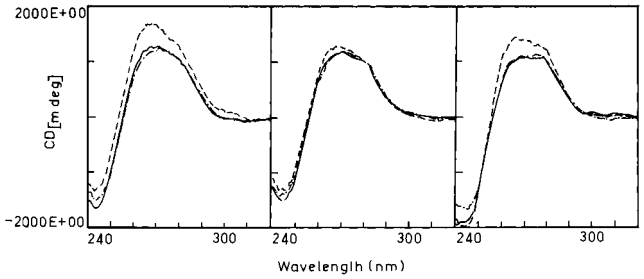

FIGURE 1: Circular dichroism spectra of oligonucleotides and complexes with wild-type λ-repressor and Y210C λ-repressor: (—) free oligonucleotides, (- - -) wild-type repressor/double-operator-site complex, (-•-) Y210C λ-repressor. The left panel shows the spectra of the O_R1-O_R2 complexes, the middle panel shows the spectra of the O_R2-O_R3 complexes, and the right panel shows the spectra of the O_L2-O_L3 complexes. The measurements were performed in 0.1 M potassium phosphate buffer, pH 8.0, at ambient temperature (25 ± 1 °C).

O_L3, respectively. Each panel contains three spectra: one of the free oligonucleotide, one of the oligonucleotide/wild-type λ-repressor mixture in a molar ratio of 1:4 (monomer), and another of the oligonucleotide/noncooperative mutant λ-repressor Y210C (which would lack the protein−protein contact) mixture in a molar ratio of 1:4 (monomer) (*18*). Y210C is a cooperative mutant isolated by Youderian and co-workers (*25*) from a biological screen to preserve DNA binding. Thus, it is likely that the mutation does not cause any significant reduction in DNA binding ability. Ackers and co-workers have also studied a noncooperative mutant in this position, Y210H (*26*, *27*). They have also shown that this mutant carries no significant DNA binding defect. This suggests that, if any, Y210C has very little defect in DNA binding and at micromolar concentrations should be largely bound to any of the naturally occurring operator sites. For O_R1-O_R2, the oligonucleotide spectrum in the complex is significantly more intense than that of the free oligonucleotide. When Y210C mutant λ-repressor is used instead of the wild-type repressor, the above-mentioned enhancement of the CD spectrum is abolished, suggesting that the enhancement originates from DNA distortion in the complex that has two cooperatively bound repressor molecules. A similar conclusion was drawn for operator sites that are 180° out of phase (*8*). For O_R2-O_R3, however, addition of either wild-type λ-repressor or the Y210C mutant produces only small changes in the CD spectra, suggesting less distortion of the DNA, if any. The oligonucleotide containing O_L2-O_L3 again shows a CD spectrum that is enhanced in the wild-type λ-repressor complex than in the free state, suggesting significant DNA distortion. As in the case of O_R1-O_R2, the Y210C mutant fails to elicit a similar response.

There are many ways to detect conformational changes in proteins. Fluorescence spectroscopy stands out as one of the foremost, as the molecule can be studied at nanomolar concentrations (*28*). This is particularly important in this case, since such weak and cooperative complexes are difficult to crystallize or study at higher concentrations needed for NMR. We have previously shown that binding of λ-repressor to the operator O_R1 at its N-terminal domain causes a conformational change in the C-terminal domain (*17*). This change is responsible for alteration of the nature of dimer−dimer contact (*8*). In the natural context, all operator sites within the left and right operators of bacteriophage λ are different

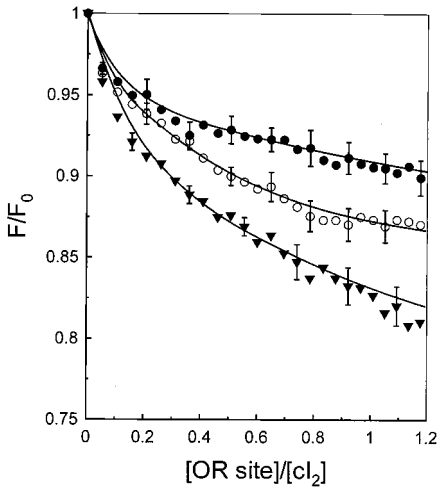

FIGURE 2: Quenching of λ-repressor tryptophan fluorescence by three single-operator sites, O_R1 (●), O_R2 (▼), and O_R3 (○). Fluorescence was measured by titrating 1 μM repressor with duplex oligonucleotides containing the appropriate sequence of the operator site in 0.1 M phosphate buffer, pH 8.0. The excitation wavelength was 295 nm, and the emission wavelength was 340 nm. The experiments were repeated three or more times. Error bars are shown for only every three or four points to avoid clutter.

with respect to sequence, although they have some homology. It is possible that different nucleic acid sequences may change the conformation of the λ-repressor differentially. This may create new possibilities for recognition of different DNA sequences. Figure 2 shows the titration of λ-repressor with oligonucleotides containing single O_R1, O_R2, and O_R3 operator sites. Although quenching of tryptophan fluorescence is observed in all cases, the extent of fluorescence quenching varies significantly. Oligonucleotide containing the O_R1 sequence produces the least fluorescence quenching ($F_\infty = 0.907 \pm 0.0023$), whereas O_R2 quenches the most ($F_\infty = 0.813 \pm 0.005$). The oligonucleotide containing O_R3 quenches the tryptophan fluorescence in-between ($F_\infty = 0.868 \pm 0.0023$). We conclude that this difference is statistically significant. We have also mixed the three operators with the λ-repressor in stoichiometric amounts (i.e., 1:1 molar ratio of operator to repressor dimer) and measured the fluorescence. In these experiments too, the O_R2 showed the maximum quenching followed by O_R3 and O_R1, suggesting that the observed differences reflect different protein conformations and do not arise from any methodological variability.

Conformational changes induced by different operator sites were evident from tryptophan fluorescence quenching. A more powerful method of investigating the different environments of the tryptophan residues in complexes with different operator sites is by collisional quenching. Acrylamide has been used widely as a collisional quencher of tryptophan fluorescence, thus providing information on the accessibility of tryptophans (*19*, *29*). Figure 3 shows the Stern−Volmer plot of acrylamide quenching of tryptophan fluorescence of λ-repressor, bound to single operator sites O_R1, O_R2, and O_R3. The data were fitted to a composite Stern−Volmer equation with two quenchable components, and the two derived Stern−Volmer constants are reported in Table 1. When the same data were fitted to a Stern−Volmer equation with three quenchable components, the best fit always converged to two components. This suggests that two

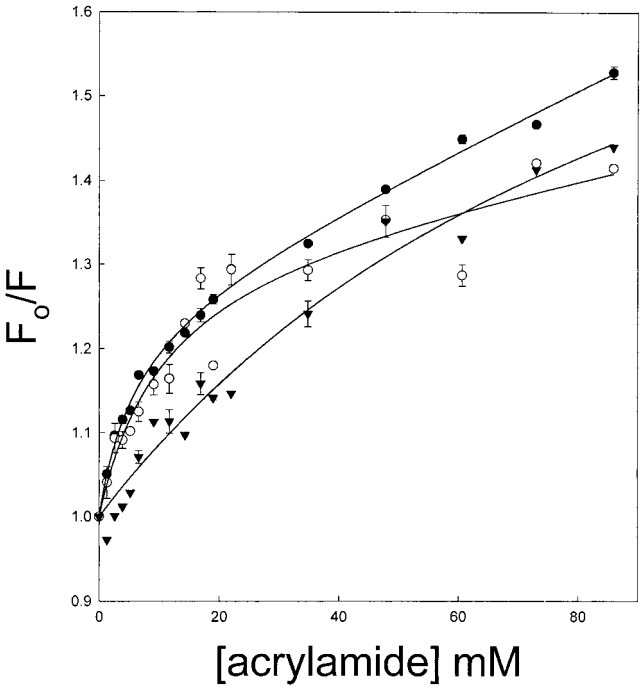

FIGURE 3: Stern−Volmer plot of acrylamide quenching of λ-repressor bound to single-operator sites O_R1 (●), O_R2 (▼), and O_R3 (○). The solid lines are the best-fit lines to the Stern−Volmer equation. The measurements were done at 1 μM protein concentration in 0.1 M potassium phosphate buffer, pH 8.0. The observed fluorescence was corrected for the inner filter effect as described in the Materials and Methods. The excitation wavelength was 295 nm, and the emission was measured at 340 nm. The experiments were repeated three or more times.

Table 1: Stern−Volmer Constants of λ-Repressor Bound to Different Single- and Double-Operator Sites

	K_{SV1} (M^{-1})	F_1	K_{SV2} (M^{-1})	F_2
O_R1	214 ± 15	0.2 ± 0.015	2.51 ± 0.26	0.78 ± 0.017
O_R2	31.5 ±10.1	0.48 ± 0.1	0.35 ± 0.3	0.52 ± 0.1
O_R3	129 ± 71	0.25±.04	1.01 ± 0.31	0.75 ± 0.04
O_R1-O_R2	126 ± 11	0.16 ± 0.01	2.6 ± 0.08	0.84 ± 0.01
O_L2-O_L3	107 ± 46	0.16 ± 0.02	2.19 ± 0.4	0.84 ± 0.02
O_R2-O_R3	47.8 ± 5	0.67 ± 0.05	0	0.33 ± 0.05
O_R2-O_R3/Y210C	193 ± 75	0.24 ± 0.07	7.27 ± 1.65	0.76 ± 0.07

quenchable components are necessary and sufficient to describe the quenching curves. Clearly the Stern−Volmer plots are different for O_R2 and similar, but not identical, for O_R1 and O_R3, thus underlining the conformational differences of repressor molecules bound to different operator sites. Although one should not draw conclusions about the change of accessibility of tryptophans in the absence of lifetime data, the change in Stern−Volmer constants does reflect a change in tryptophan environments. These results clearly support the operator binding experiments described above. It is interesting to note that Ackers and co-workers have reported that salt dependence of O_R2 binding is significantly different from that of O_R1 and O_R3 (*30*).

The conformational differences of the λ-repressor, when bound to different operator sites, suggest a fine-tuning of the orientations of the operator-site-bound dimers in the different pairs of operator sites. Additionally, the necessity of protein−protein contact in cooperatively bound dimers may force further changes in the protein conformation. We have thus measured the fluorescence quenching of λ-repressor, induced by binding to pairs of operator sites. Figure 4 shows the quenching profile of λ-repressor upon titration with oligonucleotides containing O_R1-O_R2, O_R2-O_R3, and O_L2-O_L3. Remarkably, the oligonucleotides containing O_R1-O_R2 and O_L2-O_L3 quenched the fluorescence much less than the one containing O_R2-O_R3. The extent of quenching by the O_R1-O_R2-containing oligonucleotide is lower than the quenching by either O_R1 or O_R2 alone. This clearly suggests that the conformations of the proteins are different in the cooperatively bound complex than in the single-operator-bound situation. In the case of O_R2-O_R3, however, the extent of quenching is roughly the average of quenching induced by the two constituent single operators. We stress here that because of the complexity of the fluorescence spectra of a three-tryptophan protein, it is not appropriate to conclude that there is no additional conformational change involved in the cooperative complex formation at O_R2-O_R3 from single-wavelength quenching data. The change, if any, is of a nature different from that in case of O_R1-O_R2 (see later). Thus, the difference of the extent of quenching induced by O_R1-O_R2 and O_R2-O_R3 clearly suggests that not only is the protein conformation different in the cooperative complex when compared to the single-operator-bound situation, but also the protein conformations induced by the protein−protein contact are different in different cooperative complexes.

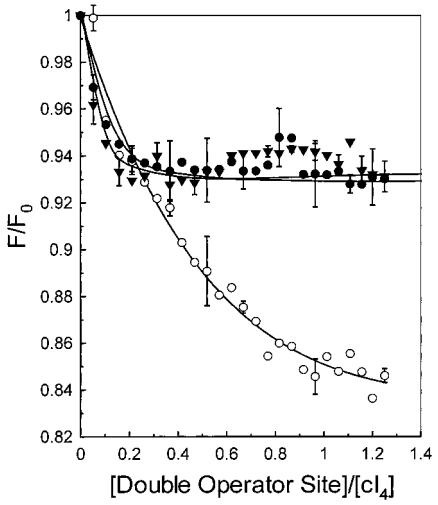

FIGURE 4: Quenching of λ-repressor tryptophan fluorescence by three double-operator sites, O_R1-O_R2 (●), O_R2-O_R3 (○), and O_L2-O_L3 (▼). The experiment was carried out by titrating 1 μM λ-repressor with increasing concentrations of oligonucleotides containing double-operator sites. The excitation wavelength was 295 nm, and the emission was monitored at 340 nm. The solution conditions were 0.1 M potassium phosphate buffer, pH 8.0, at 25 °C. The experiments were repeated three or more times. Error bars are shown for only every three or four points to avoid clutter.

As in the case of the single operators, acrylamide quenching can be used to further probe the conformational differences between the different cooperative complexes.[2] Figure 5 shows the Stern−Volmer plot of the acrylamide quenching of tryptophan fluorescence of λ-repressor, when bound to the three different pairs of double-operator sites. The Stern−Volmer constants derived as before are reported in Table 1.

[2] The term "cooperative complex" is defined as a mixture of 0.25 μM oligonucleotide containing two operator sites in natural context with 1 μM wild-type repressor. This assumes that the repressor molecules are bound to the two operator sites cooperatively.

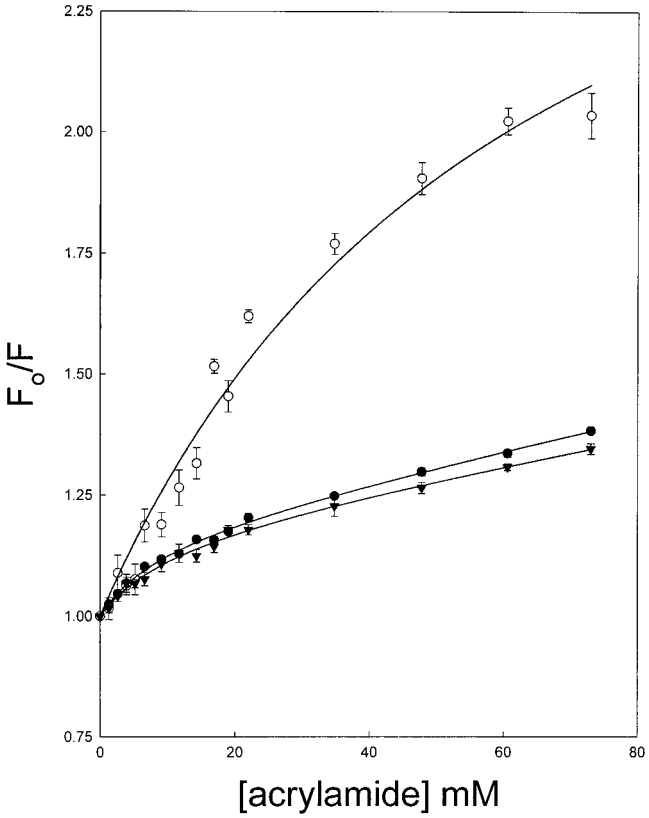

FIGURE 5: Stern−Volmer plot of acrylamide quenching of λ-repressor bound to double-operator sites O_R1-O_R2 (●), O_L2-O_L3 (▼), and O_R2-O_R3 (○). The solid lines are the best-fit lines to the Stern−Volmer equation. The solution conditions were the same as those for Figure 4.

The repressor bound to O_R1-O_R2 and O_L2-O_L3 is far less quenchable by acrylamide than the repressor bound to O_R2-O_R3. This is evidenced by the fact that the highly quenchable component of the latter is almost 67% of the total fluorescence, whereas it is only 16% of the total fluorescence of the former two. Interesting points emerge when we compare the quenching profiles of the single-operator-bound repressors and the cooperative complexes. The O_R2-bound repressor has 50% of its fluorescence not quenchable at all. However, in the O_R1-O_R2 cooperative complex, there appears to be no nonquenchable fraction. This strongly indicates that the cooperative complex formation in the O_R1-O_R2 double-operator sites involves further conformational change in the repressor that results in increased accessibility of the tryptophan(s) to acrylamide. Similar differences are found for the O_R2-O_R3-bound cooperative complex. As a control, we have performed acrylamide quenching of O_R2-O_R3/Y210C repressor complex. Previously we have shown that the Y210C quenching pattern with acrylamide- and operator-induced conformational change is similar to that of the wild-type repressor (*18*). The Stern−Volmer constants and the quenchable fractions are significantly different from those of the corresponding wild-type complex, suggesting additional conformational change upon cooperative complex formation.

Bis-ANS has been widely used as a probe of apolar binding sites of proteins. Its emission maximum and quantum yield are sensitive to the polarity of its environment. At low concentrations (around 10 μM), bis-ANS binds to protein without noticeable denaturation of the protein, whereas at higher concentrations significant denaturation is observed (*31*). Although exact binding sites of bis-ANS are not known, it is likely that it binds to domain−domain interfaces, which are significantly more apolar than the surfaces. We have previously shown that bis-ANS binds to the C-terminal domain of the λ-repressor and can report on the operator-induced conformational change. We have thus studied the binding of bis-ANS to the λ-repressor/double-operator-site cooperative complexes. Figure 6 shows the plot of the fluorescence ratio of double-operator complexes and free repressor as a function of bis-ANS concentration. Clearly, O_R1-O_R2 and O_R2-O_R3 complexes follow approximately the same pattern, but O_L2-O_L3 is significantly different from the other two, thus suggesting that O_L2-O_L3/λ-repressor complex conformation is distinctly different from the other two with respect to bis-ANS binding. As a control, we have titrated a complex of O_R1-O_R2/Y210C repressor and O_L2-O_L3/Y210C repressor with increasing concentrations of bis-ANS, and the results are shown in Figure 6. In contrast to the cooperative complexes where the fluorescence ratio shows a decreasing trend with increasing bis-ANS concentration, the fluorescence ratio increases with increasing bis-ANS concentration. This indicates that the formation of cooperative contacts leads to a further conformational change that may be different in different double-operator-site pairs.

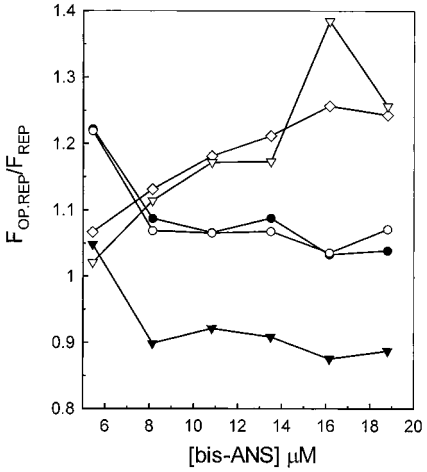

FIGURE 6: Binding of bis-ANS to double-operator site/λ-repressor cooperative complexes. The *y*-axis represents the fluorescence ratio of O_R1-O_R2 (●), O_L2-O_L3 (▼), and O_R2-O_R3 (○) and the free repressor. The figure also shows titration of O_R1-O_R2/Y210C (◇) and O_L2-O_L3/Y210C (▽) complexes with bis-ANS. Free repressor and the three cooperative complexes were titrated with increasing concentrations of bis-ANS, and the fluorescence was determined. All the experiments were done in a single day to avoid day to day variation. Similar experiments were carried out with Y210C repressor as the control. The repressor concentration was 1 μM, and oligonucleotide concentrations were 0.25 μM.

DISCUSSION

Selective binding of proteins to one or more DNA sequences is the first step in the regulation of gene expression as well as in many other phenomena involving the DNA. A number of crystal structures of a DNA-binding protein complexed to its binding site have been published. X-ray crystal structure and NMR study of such complexes have elucidated some of the atomic interactions that characterize specific binding of a protein to a nucleic acid sequence (*1*−*3*, *32*−*37*). Much less is known about the recognition of

multipartite operators by DNA-binding proteins, although an increasing number of proteins are thought to bind to more than one sequence in the DNA. One of the best known multipartite operator systems is that of the operator sites of bacteriophage λ. The bacteriophage λ has four naturally occurring pairs of sites (O_R1-O_R2, O_R2-O_R3, O_L1-O_L2, and O_L2-O_L3), to which the repressor binds cooperatively. These pairs of sites have different intervening distances and relative orientations. There are several ways by which the repressor tetramer can compensate for these differences: (i) the intervening DNA sequence may be distorted, (ii) the protein conformation may be changed, either in response to binding to different nucleic acid sequences or in response to energetic requirements of bridging different distances and orientations, (iii) the protein−DNA interface may be changed, and (iv) protein−protein interfaces may be different. Each of these elements has to be studied to arrive at a complete picture.

In this paper, we have shown that in two of the pairs of sites, O_R1-O_R2 and O_L2-O_L3, cooperative binding of repressor leads to a very significant distortion of the intervening DNA as monitored by circular dichroism. Strahs and Brenowitz (*38*) have studied the conformation of the O_R DNA by footprinting, and the conclusions are in agreement with this result. However, in the case of O_R2-O_R3, very little DNA distortion is observed using circular dichroism. Both Merabet and Ackers (*39*) and Strahs and Brenowitz (*38*) have concluded that some of the effects of adjacent site binding are transmitted through the intervening DNA. The changes in circular dichroism spectra that are seen upon binding are largely a result of protein−protein contact (as it disappears in the complex with the noncooperative mutant). However, some small differences may be observed between the noncooperative mutant complex and the free oligonucleotide. Whether such small differences reflect the structural changes postulated by Merabet and Ackers (*39*) and Strahs and Brenowitz (*38*) remains to be investigated.

Clearly, the different distances of separation as well as the different orientations, though significant, are not necessarily the only factors for cooperative binding. Previously, we have shown that single-operator-site binding to λ-repressor induces a conformational change in the C-terminal domain of the protein (*17*). Later studies have shown that one of the major effects of such a conformational change is the modulation of protein−protein interaction mediated partly by the C-terminal tail region (*8*). One of the surprises of this study is that the sequence of the operator site fine-tunes the conformational change in the C-terminal domain, thus introducing another layer of regulation. Such different conformational changes induced by different operator sites may place the interacting patch of the C-terminal domain at different orientations, thus necessitating different conformations of the intervening DNA. We note that on the basis of their study of variant *lac* operators Frank et al. (*7*) have reached conclusions that operator sequences have a profound effect on the bound conformation of *lac* repressor.

All three tryptophans of λ-repressor are located in the C-terminal domain, thus providing us with a powerful probe of the conformation of the C-terminal domain. In addition, bis-ANS, a noncovalent fluorescence probe, also binds selectively to the C-terminal domain (*17*). As stated above, these probes indicate that (a) the conformations of the C-terminal part are different in complexes with different single operators and (b) the λ-repressor/double-operator cooperative complexes have significantly different structures. Acrylamide quenching experiments suggest that the conformation of λ-repressor bound to O_R2-O_R3 and O_R1-O_R2 is significantly different from that of λ-repressor bound to the single-operator sites. This suggests an additional conformational change takes place in the protein upon establishment of protein−protein contacts in the cooperative complex.

From the above discussion it is clear that the recognition of multipartite operators by λ-repressor may require distortion of the intervening DNA, different conformations of the protein as dictated by the nucleic acid sequence it is bound to, and further changes in protein conformation upon cooperative complex formation. Whether the third and the fourth possibilities mentioned above occur in these multipartite operator systems is not known. However, we find it instructive that quantitative footprinting titrations by Ackers and co-workers have indicated that there are differential effects of some mutations on cooperativity depending on the nature of the double-operator sites used (*26*, *27*, *40*). This could be the result of having different protein−protein interfaces, although further structural studies are needed to rule out other possibilities. In conclusion, we observe that the recognition of multipartite operators by λ-repressor involves DNA distortion and DNA sequence dependent and sequence independent conformational changes. To our knowledge this is the first report that different naturally occurring DNA sequences can change bound conformations of the regulatory protein differently. How each of these elements is used depends on the sequence, separation, and orientations of the binding sites.

REFERENCES

1. Beamer, L. J., and Pabo, C. O. (1992) *J. Mol. Biol. 227*, 177−96.
2. Brennan, R. G., Roderick, S. L., Takeda, Y., and Matthews, B. W. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 8165−9.
3. Kissinger, C. R., Liu, B. S., Martin-Blanco, E., Kornberg, T. B., and Pabo, C. O. (1990) *Cell 63*, 579−90.
4. Klemm, J. D., Rould, M. A., Aurora, R., Herr, W., and Pabo, C. O. (1994) *Cell 77*, 21−32.
5. Wolberger, C., and Harrison, S. C. (1987) *J. Mol. Biol. 196*, 951−4.
6. Tan, S., and Richmond, T. J. (1998) *Nature 391*, 660−6.
7. Frank, D. E., Saecker, R. M., Bond, J. P., Capp, M. W., Tsodikov, O. V., Melcher, S. E., Levandoski, M. M., and Record, M. T., Jr. (1997) *J. Mol. Biol. 267*, 1186−206.
8. Bandyopadhyay, S., Mukhopadhyay, C., and Roy, S. (1996) *Biochemistry 35*, 5033−40.
9. Adhya, S. (1989) *Annu. Rev. Genet. 23*, 227−50.
10. Schleif, R. (1992) *Annu. Rev. Biochem. 61*, 199−223.
11. Majumdar, A., and Adhya, S. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 6100−4.
12. Aki, T., Choy, H. E., and Adhya, S. (1996) *Genes Cells 1*, 179−88.
13. Dunn, T. M., Hahn, S., Ogden, S., and Schleif, R. F. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 5017−20.
14. Ptashne, M. (1986) *Nature 322*, 697−701.
15. Ptashne, M. (1992) *A genetic switch*: *Phage lambda and higher organisms*, Cell Press, Boston.
16. Senear, D. F., Brenowitz, M., Shea, M. A., and Ackers, G. K. (1986) *Biochemistry 25*, 7344−54.
17. Saha, R., Banik, U., Bandopadhyay, S., Mandal, N. C., Bhattacharyya, B., and Roy, S. (1992) *J. Biol. Chem. 267*, 5862−7.

18. Deb, S., Bandyopadhyay, S., and Roy, S. (1998) *Protein Eng. 11*, 481−487.
19. Bandyopadhyay, S., Banik, U., Bhattacharyya, B., Mandal, N. C., and Roy, S. (1995) *Biochemistry 34*, 5090−7.
20. Flashner, Y., and Gralla, J. D. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8968−72.
21. Kramer, H., Niemoller, M., Amouyal, M., Revet, B., von Wilcken-Bergmann, B., and Muller-Hill, B. (1987) *EMBO J. 6*, 1481−91.
22. Sasse-Dwight, S., and Gralla, J. D. (1988) *J. Mol. Biol. 202*, 107−19.
23. Choy, H. E., and Adhya, S. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 11264−8.
24. Cantor, C. C., and Schimmel, P. R. (1980) *Biophysical Chemistry*, W. H. Freeman, San Fransisco.
25. Benson, N., Adams, C., and Youderian, P. (1994) *Mol. Microbiol. 11*, 567−579.
26. Burz, D. S., and Ackers, G. K. (1994) *Biochemistry 33*, 8406−16.
27. Burz, D. S., Beckett, D., Benson, N., and Ackers, G. K. (1994) *Biochemistry 33*, 8399−405.
28. Beechem, J. M., and Brand, L. (1985) *Annu. Rev. Biochem. 54*, 43−71.
29. Lakowicz, J. R. (1983) *Principles of Fluorescence Spectroscopy*, Plenum, New York.
30. Koblan, K. S., and Ackers, G. K. (1991) *Biochemistry 30*, 7822−7.
31. Shi, L., Palleros, D. R., and Fink, A. L. (1994) *Biochemistry 33*, 7536−46.
32. Albright, R. A., and Matthews, B. W. (1998) *J. Mol. Biol. 280*, 137−51.
33. Arrowsmith, C. H., Pachter, R., Altman, R. B., Iyer, S. B., and Jardetzky, O. (1990) *Biochemistry 29*, 6332−41.
34. Otting, G., Qian, Y. Q., Billeter, M., Muller, M., Affolter, M., Gehring, W. J., and Wuthrich, K. (1990) *EMBO J. 9*, 3085−92.
35. Otwinowski, Z., Schevitz, R. W., Zhang, R. G., Lawson, C. L., Joachimiak, A., Marmorstein, R. Q., Luisi, B. F., and Sigler, P. B. (1988) *Nature 335*, 321−9.
36. Somers, W. S., and Phillips, S. E. (1992) *Nature 359*, 387−93.
37. Wolberger, C., Dong, Y. C., Ptashne, M., and Harrison, S. C. (1988) *Nature 335*, 789−95.
38. Strahs, D., and Brenowitz, M. (1994) *J. Mol. Biol. 244*, 494−510.
39. Merabet, E., and Ackers, G. K. (1995) *Biochemistry 34*, 8554−8563.
40. Burz, D. S., and Ackers, G. K. (1996) *Biochemistry 35*, 341−50.

BI9919955