# Dimer−Dimer Interfaces of the λ-Repressor Are Different in Liganded and Free States†

Sumita Bandyopadhyay, Chhanda Mukhopadhyay, and Siddhartha Roy*

*Department of Biophysics, Bose Institute, P 1/12 C.I.T. Scheme VII M, Calcutta 700 054, India*

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ABSTRACT: λ-Repressor dimers associate in solution to form tetramers and higher order structures. Dimer−dimer contact is also crucial in cooperative binding to adjacent operators. Fluorescence quenching studies indicate that the tryptophan 230 environment is significantly different in unliganded and adjacent operator-bound tetramers. Acrylodan attached to Cys 235, in a mutant F235C repressor, is also in different environments in the unliganded and adjacent operator bound tetramers. Thermodynamics of protein association, measured by fluorescence anisotropy, indicate that, whereas free repressor dimer association is strongly enthalpy driven, the single-operator ($O_R1$)-bound repressor dimer association is largely entropy driven with little change in enthalpy. Single-operator-bound dimer association to the corresponding tetramer does not lead to any significant change in tryptophan 230 environment, as was seen in the case of the free repressor. Data are also presented to support the contention that, under the conditions of this study, the free repressor association is predominantly from dimer to tetramer and then to octamer, unlike the dimer to octamer transition observed under a different condition. The results presented here point toward the conclusion that the λ-repressor dimer−dimer interface is significantly different in the free and the operator-bound states and that operator binding plays a crucial role in changing the nature of the dimer−dimer interface.

It is increasingly becoming clear that protein−protein interaction between the regulatory proteins is crucial in regulating gene expression. Many prokaryotic and eukaryotic systems are now known in which heterologous and homologous protein−protein contacts play pivotal roles in regulation of gene expression (Guerente *et al*., 1982; Adhya, 1989; Ishiama, 1993; Dunn *et al*., 1984; Schleif, 1992).

The lytic−lysogenic switch of bacteriophage λ is mainly regulated by two proteins: cro and λ-repressor (Ptashne, 1992). This regulatory system has many features of other complex gene regulatory systems and has been studied intensively, from genetic and structural points of view, as a model regulatory system (Shea & Ackers, 1985; Sauer *et al*., 1990). The regulatory system consists of three adjacent operator sites, $O_R1$, $O_R2$, and $O_R3$, to which the λ-repressor binds with alternate pairwise cooperativity (Johnson *et al*., 1979; Senear *et al*., 1986). The cooperative binding of the λ-repressor to these adjacent operators results from contact between the operator-bound repressor dimers (Hochschild & Ptashne, 1986). Although it is fairly certain that the protein−protein contact occurs through the C-terminal domains of the bound dimers, further localization has been only partially successful (Whipple *et al*., 1994; Benson *et al*., 1993).

λ-Repressor dimer also oligomerizes in solution. It is generally believed that this oligomerization is mediated through the C-terminal domain as well (Pabo *et al*., 1979). The aggregation behavior of several non-cooperative mutants has been studied (Burz & Ackers, 1994). These results, along with our recent spectroscopic and chemical modification study, have given some idea about the nature of this dimer−dimer interface (Bandyopadhyay *et al*., 1995). Among the non-cooperative mutants that have been studied in detail, Serine 228 to Asparagine (S228N) appears very interesting. It is weak in monomer−dimer association. Its initial characterization as a non-cooperative mutant (Hochschild & Ptashne, 1988) has now been disputed by Burz and Ackers (1994), who had detected wild-type-like cooperative interaction. They have indicated that this mutant may, however, be defective in unliganded oligomer formation in solution. This has raised an interesting possibility, that the protein−protein interface in unliganded tetramer and adjacent operator ($O_R1-O_R2$) bound tetramers may be different. Clearly, if the protein−protein interfaces between regulatory proteins are context dependent, another degree of complexity may be added to the process of regulation. Thus, it would be of considerable importance to investigate the structure and energetics of adjacent operator-bound repressor dimers. In this article we describe spectroscopic studies of oligonucleotide fragments containing $O_R1$ and $O_R2$ and their complexes with the λ-repressor and discuss implications regarding the nature of the dimer−dimer interface.

## EXPERIMENTAL PROCEDURES

*Materials*. Acrylamide, isopropyl β-D-thiogalactopyranoside (IPTG),[1] polyethylene imine, and PMSF were purchased from Sigma Chemical Co. (St. Louis, MO). Bacto-tryptone, bacto-agar, and yeast extract were purchased from Difco

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* To whom all correspondence should be addressed. FAX: 91-33-334-3886. E-mail: siddarth@boseinst.ernet.in.

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[1] Abbreviations: FITC, fluorescein 5-isothiocyanate; β-ME, 2-mercaptoethanol; DMF, *N*,*N*-dimethylformamide; acrylodan, 6-acryloyl-2-(dimethylamino)naphthalene; IPTG, isopropyl β-D-thiogalactoside; CD, circular dichroism; dansyl chloride, 5-(dimethylamino)naphthalene-1-sulfonyl chloride.

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Laboratories (Detroit, MI). $\beta$-Mercaptoethanol and glycerol were purchased from Aldrich Chemical Co. (Milwaukee, WI). Acrylodan and FITC were purchased from Molecular Probes Inc. (Eugene, OR). All other reagents were of analytical grade.

*Repressor Isolation.* $\lambda$-Repressor was isolated from a strain of *Escherichia coli* RR1 15 Δlac Z carrying a plasmid pEA305 which contains the wild-type cI gene under the control of *tac* promoter. The details of the purification procedure has been given in Saha *et al*. (1992). The native repressor concentration was determined using $E^{1\%}_{280} = 11.3$. Molar concentration of the repressor was always calculated in terms of monomer subunit, unless mentioned otherwise. For all studies, the repressor was dialyzed against 0.1 M potassium phosphate buffer, pH 8.0, unless specifically mentioned otherwise.

*Synthesis of Oligonucleotides*. Oligonucleotides were synthesized, with the terminal trityl group on, in an Applied Biosystems DNA synthesizer (model 381A). The oligonucleotides were cleaved from the column and deprotected by 30% ammonium hydroxide. This was then precipitated by *n*-butanol, and then the pellet containing the oligonucleotide was dried under vacuum and redissolved in 0.1 M triethylammonium acetate buffer, pH 7.0. These were then purified according to Saha *et al*. (1992), using reverse phase HPLC ($\mu$-Bondapak C-18 column). The oligonucleotides were then detritylated and transferred to an aqueous buffer, mixed in 1:1 molar proportions, and annealed. Concentrations were calculated from $A_{260}$, using extinction coefficients which were calculated assuming an extinction coefficient for purine to be $14 \times 10^3$ and that for pyrimidine to be $7 \times 10^3$ (Roy *et al*., 1986). The calculated extinction coefficients for oligonucleotides are as follows: $O_R1$ = 5′-GTACCTCTGGCGGTGATAG-3′ and its complementary sequence, $\epsilon = 40 \times 10^4$ $M^{-1}$ $cm^{-1}$; wild-type $O_R1-O_R2$ = 5′-CCTATCACCGCCAGAGGTAAAATAGTCAACACGCACGGTGTTACCA-3′ and its complementary sequence, $\epsilon = 99.4 \times 10^4$ $M^{-1}$ $cm^{-1}$; and control $O_R1-O_R2$ = 5′-TATCACCGCCAGAGGTAAACCATACCGTCAACACGCACGGTGTTA-3′ and its complementary sequence, $\epsilon = 94.5 \times 10^4$ $M^{-1}$ $cm^{-1}$. Henceforth, we will refer to the latter two sequences as wild-type $O_R1-O_R2$ and control $O_R1-O_R2$, respectively.

*Chemical Modification.* Labeling of $\lambda$-repressor with dansyl chloride was done according to Banik *et al*. (1993) by incubating 0.5 mg of repressor/mL in 0.24 M phosphate buffer, pH 8.0, with 10-fold molar excess of dansyl chloride (in DMF) at 25 °C for 15 min. The reaction was quenched by adding tris-HCl, pH 7.8, to a final concentration of 1 mM. It was then dialyzed against 0.1 M phosphate buffer, pH 8.0. The incorporation ratio was found to be $1.1 \pm 0.2$.

Acrylodan modification of F235C mutant repressor was done in 0.1 M phosphate buffer, pH 8.0 F235C mutant repressor, 10 $\mu$M, dialyzed against the same buffer was incubated with 1.0 mM acrylodan (added as DMF solution in such a way that the final DMF concentration did not exceed 1%) for 30 min at 25 °C. The reaction was quenched with 10 mM $\beta$-ME and dialyzed extensively against 0.1 M Phosphate buffer, pH 8.0, to remove the excess reagent. The final incorporation was determined using $\epsilon_{360} = 18\,000$ $M^{-1}$ $cm^{-1}$ (Haugland, 1992), and the protein estimation was done according to the method of Bradford (1976).

*Fluorescence Methods*. Acrylamide quenching of tryptophan fluorescence was done by adding a freshly prepared solution of a high concentration of three times re-crystallized acrylamide in 0.1 M potassium phosphate buffer, pH 8.0, to the protein solution. All fluorescence values were corrected for volume changes and inner filter effect, the latter correction being made according to the following formula:

$$F_{corr} = F_{obs}\ \text{antilog}[(A_{ex} + A_{em})/2]$$

where $A_{ex}$ is the absorbance at the excitation wavelength, $A_{em}$ is the absorbance at the emission wavelenght, $F_{corr}$ is the corrected fluorescence, and $F_{obs}$ is the observed fluorescence. Absorbances at the excitation and emission wavelengths were measured before and after a titration, and the intermediate values were obtained by interpolation.

Acrylodan-labeled $\lambda$-repressor at 0.5 $\mu$M (labeled and wild-type repressor was mixed at 1:3 ratio) was titrated with $O_R1$, native $O_R1-O_R2$, and control $O_R1-O_R2$. Appropriate blank spectra were always subtracted from the actual spectra. Fluorescence values at 510 nm were measured and were corrected for dilution.

*Anisotropy Measurements*. Anisotropy experiments were performed using an Hitachi polarization accessory. The fluorescence intensity components ($I_{hh}$, $I_{hv}$, $I_{vv}$, $I_{vh}$), in which the subscripts refer to the horizontal (h) and vertical (v) positioning of the excitation and the emission polarizers, respectively, were used to calculate the steady-state fluorescence anisotropy ($A$) according to the following equation:

$$A = (I_{hh} - GI_{hv})/(I_{hh} + 2GI_{hv})$$

where $G$ is the grating factor that corrects for wavelength dependent distortions of the polarizing system and is equal to

$$I_{vh}/I_{vv}$$

*Light Scattering.* $\lambda$-Repressor at a high concentration was Millipore filtered three times (by a syringe filter). It was then added in small aliquots into an appropriate Millipore-filtered buffer in a fluorescence cuvette under a laminar flow hood to avoid dust contamination. The covered cuvette was transferred into a fluorimeter, and the scattering was measured with both the excitation and the emission wavelengths set at 330 nm. Scan speed and band passes were set at 15 nm/min and 5 nm, respectively. All further additions were done under a laminar flow hood. The scattering intensity was recorded at each concentration. The scattering due to buffer alone was subtracted from each value.

*Circular Dichroism*. Circular dichroism measurements were done on a JASCO J600 spectropolarimeter using a 1 cm pathlength quartz cuvette. The scan speed was 120 nm per minute. 10 scans were signal averaged to increase the signal to noise ratio. The CD spectra of oligonucleotides and the oligonucleotide complexes were taken at oligonucleotide concentrations of 0.125 and 0.25 $\mu$M and protein concentrations of 0.5 and 1.0 $\mu$M, respectively. 2× stock solutions of the protein and the oligonucleotides were mixed in equal volumes by weighing in a Sartorius microbalance to minimize pipetting errors. Oligonucleotides and protein solutions at the same concentrations were prepared by mixing equal volumes of buffer and 2× solutions by weighing, as described above. The buffer-only spectrum was subtracted from the oligonucleotide spectra, and the protein-only spectrum was subtracted from the complex spectra.

*Large Zone Gel Filtration*. Large zone experiments were performed at room temperature on a Sephacryl S-200 column (60 × 0.8 cm). Flow rates were typically 0.25 mL/min, controlled by a peristaltic pump. Protein solutions, typically 25−30 mL, were applied to the column to establish a plateau in the elution profile of protein concentration vs elution volume. The centroid volume, $V_e$, provides a measure of the weight-average partition coefficient, $\sigma_w$, according to the equation below:

$$\sigma_w = (V_e - V_o)/V_i$$

where $V_o$ is the void volume, which was determined by using blue dextran, and $V_i$ is the internal volume of the column. $V_i + V_o$ was determined by using L-tryptophan. Evaluation of the elution volume for each zone requires the determination of an equivalent sharp boundary (centroid) for the leading or the trailing edges of the solute profile (Ackers, 1975; Koblan & Ackers, 1991a). The equivalent sharp boundaries were determined by drawing an expanded profile on a graph paper and then drawing a vertical line so that it divides the profile into two triangles of equal areas. The molecular weight calibration curve was done in both buffers, using known molecular weight markers (carbonic anhydrase, BSA, tubulin, yeast alcohol dehydrogenase, β-amylase, apoferritin). The elution volumes of same proteins do not differ significantly in different buffer systems. The volumes were measured by weighting the empty tube and then after the collection of eluant (fraction volumes were around 250−300 μL).

*Tetramer−Dimer Dissociation*. In a typical experiment, dansyl-labeled repressor was mixed with unlabeled repressor at a 1:9 ratio. A stoichiometric equivalent amount of single operator $O_R1$ was added to the repressor solution. The samples were then progressively diluted to various concentrations with 0.1 M phosphate buffer, pH 8.0. In all cases, the buffer blank was subtracted from each value. Signal averaging was performed to increase the signal to noise ratio. Constant temperature was maintained by circulating water. The temperature of the sample chamber was measured by dipping a thermometer into another cuvette kept in the chamber. The buffer with which the sample was diluted was also kept in that chamber. The mathematical expression for the observed anisotropy ($A$) as a function of the total protein concentration, was obtained by combining Weber average formula (Lakowicz, 1986) with the expression for tetramer−dimer equilibrium constant

$$A = \{K_d[-1 + \sqrt{(1 + 4P_o/K_d)}][2A_d + A_t[-1 + \sqrt{(1 + 4P_o/K_d)}]]\}/4P_o$$

where $A$ is the observed anisotropy, $A_d$ is the anisotropy of the dimer, $A_t$ is the anisotropy of the tetramer, $P_o$ is the total protein concentration expressed in terms of monomer, and $K_d$ is the tetramer−dimer dissociation constant.

As shown above, the resultant expression contains three unknown parameters, $K_d$, $A_d$, and $A_t$. The three unknown parameters are systematically varied within a given range, and a least-squares estimate was used to judge the quality of the fit with the experimental data. A set of parameter values that gave the minimum least-squares value was chosen as the best-fit curve.

## RESULTS

It is now fairly certain that the interactions between the DNA bound regulatory proteins are critical for the regulation of gene expression. The interaction between two protein molecules bound to DNA sequences that may be far apart may cause the intervening DNA sequences to distort or loop out (Majumdar & Adhya, 1983; Hochschild & Ptashne, 1986, 1988; Matthews, 1992; Dunn *et al*., 1984; Martin *et al*., 1986). Although the phenomenon is now well established, the energetics and structural changes associated with the process are not well understood. The measured net interaction energy of the two proteins, often expressed as cooperativity, is probably a function of (a) the protein−protein interaction energy without the constraint of being bound to the adjacent sites on the DNA, (b) the entropic gain due to proximity of the two molecules bound to the DNA, (c) the DNA distortion energy, and (d) the energy required to promote the necessary conformational change in the protein. A complete understanding of this phenomenon requires the study of these individual effects in molecular terms.

Cooperative binding of the λ-repressor to adjacent operators has been studied intensively as a model system for a regulatory complex (Brenowitz *et al*., 1986a,b, 1989; Senear *et al*., 1986; Senear & Ackers, 1990). Energetics of the cooperative interaction has been studied by Ackers and coworkers (Beckett *et al*., 1993; Senear *et al*., 1986; Koblan & Ackers, 1991b) using quantitative footprint titrations. The net cooperative interaction energy appears to be small in magnitude (approximately 2−3 kcal/mol) and temperature independent, suggesting $\Delta H$ to be approximately zero. The interaction energy of unliganded dimers has been measured in solution (i.e., dimer−tetramer association equilibrium), which is enthalpy driven with $\Delta H$ being equal to −26.6 kcal/mol (Banik *et al*., 1993). If the dimer−dimer interaction enthalpy is so different in the unliganded and the adjacent operator bound states, it may result either from (a) the different nature of the residues involved in protein−protein interaction in the two cases or (b) from the compensation of the large enthalpy gain of association of the unliganded dimers by unfavorable enthalpies of protein and DNA distortion in the complex (the enthalpy gain due to proximity effect, which is primarily an entropic effect, should be zero). If the first possibility is true, then two scenarios are possible. The large enthalpy difference may result from either (i) the change in the nature of the protein−protein interaction due to the conformational change induced in the protein by the operator binding (Saha *et al*., 1992; Bain & Ackers, 1994) or (ii) due to the spatial restriction imposed by binding to the adjacent operators or both.

To look into these questions in detail, we have investigated the energetics of association of the single isolated $O_R1$−repressor dimer complex. The association of the repressor dimer−single $O_R^1$ operator complex was measured by the same way as described in Banik *et al*. (1993), using dansylated repressor. A stoichiometric complex of repressor tetramer (dansyl-labeled) was formed at around 20 μM concentration, where the repressor is likely to be in tetrameric state (Banik *et al*., 1993; this article). The anisotropy was then measured, the complex was progressively diluted by the addition of buffer, and the anistropy was measured at

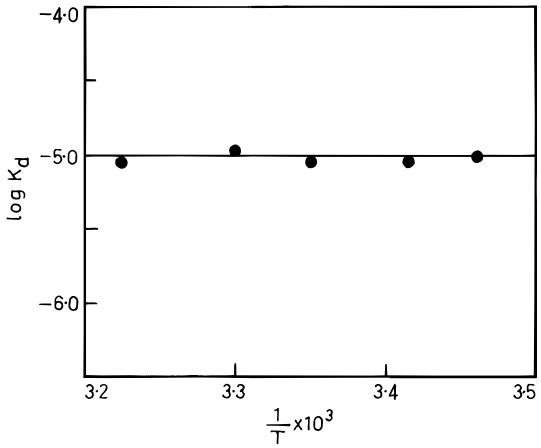


FIGURE 1: Van't Hoff plot of tetramer−dimer dissociation of dansyl-labeled $\lambda$-repressor in presence of a stoichiometric amount of $O_R1$. The dissociation at five different temperatures, 16, 20, 25, 30, and 37 °C, were determined by fluorescence anisotropy. For comparison the results were plotted in the same scale as that of the unliganded repressor in Banik *et al*. (1993). The solution conditions were 0.1 M potassium phosphate buffer, pH 8.0. The excitation wavelength was 340 nm, and the emission wavelength was 520 nm.

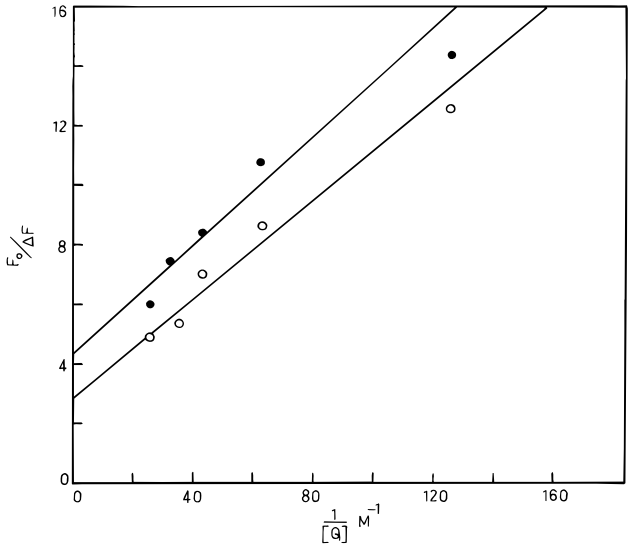


FIGURE 2: Lehrer plot of acrylamide quenching of 17 $\mu$M (●) $\lambda$-repressor and 17 $\mu$M $\lambda$-repressor in presence of a stoichiometric amount of single-operator $O_R1$ (○), i.e., 1:1 molar ratio of operator: repressor dimer. The solution conditions were 0.1 M phosphate buffer pH 8.0, 25 °C. The excitation and the emission wavelengths were 295 and 340 nm, respectively. The excitation and the emission band passes were set at 5 nm.

each complex concentration. The high affinity of the single operator for the dimeric repressor ensures that the protein does not dissociate from the operator in this concentration range. The use of a protein concentration in the micromolar range also rules out any significant dimer−monomer dissociation (Koblans & Ackers, 1991a). The obtained anisotropy versus the repressor−$O_R1$ complex concentration profile is similar to that of Banik *et al*. (1993) (data not shown). Nonlinear least-squares fits to a tetramer−dimer dissociation model (see later) yielded the tetramer−dimer dissociation constant of the repressor−$O_R1$ complex. Figure 1 shows the van't Hoff plot of tetramer−dimer dissociation of the repressor-dimer−$O_R1$ complex. The plot is virtually parallel to the temperature axis (*X*-axis) indicating near zero $\Delta H$. The association is thus primarily entropy driven, in contrast to the highly enthalpy driven association of the unliganded repressor (Banik *et al*., 1993). Thus, it is likely that the operator-induced conformational change, which has been seen before (Saha *et al*., 1992; Bain & Ackers, 1994), causes significant change in the nature of the protein−protein contact.

If the thermodynamics of the protein−protein association is different in the unliganded and the single operator-bound repressors, then the nature of the residues involved may also be significantly different. Previously, we have shown that a tryptophan residue may be involved in the dimer−dimer association in the unliganded state (Bandyopadhyay *et al*., 1995). In order to obtain structural information on the above mentioned states, we have examined the acrylamide quenching of the unliganded tetramer and the tetramer containing two bound single operators. Since acrylamide may have an effect on the operator−repressor equilibrium, we have attempted to measure the stability of $O_R1$−repressor complex as a function of the acrylamide concentration. We have synthesized an $O_R1$ operator (29 mer) with an amino link, 5′-xCTATTTTACCTCTGGCGGTGATAATGGTT-3′ and its complementary sequence, where x stands for the hexyl amino group at the 5′-end, and labeled it with FITC. The purified labeled operator was used to measure repressor binding by fluorescence anisotropy. Similarly labeled operator fragments have been used to derive the binding isotherms for the trp-repressor (LeTilly & Royer, 1993). Upon binding of the repressor to this FITC labeled operator fragment, we have observed increases in anisotropy which are not unlike those seen in the case of the trp-repressor. The anisotropy value of the operator repressor complex remains stable up to about 0.1 M acrylamide. Beyond that concentration, a slow decrease is seen (data not shown). Previously, we have shown that the unliganded protein association is unaffected even at 0.5 M acrylamide (Bandyopadhyay *et al*., 1995). Hence, we have restricted ourselves to quenching up to 0.1 M acrylamide in all the repressor-operator complexes studied by acrylamide quenching. Figure 2 shows the Lehrer plots (Lehrer, 1971) of acrylamide quenching of unliganded and $O_R1$−repressor complex at 17 $\mu$M protein concentration. The initial part of the Lehrer plot of $O_R1$−repressor complex has a $K_{sv}$ of 26 M$^{-1}$, and, when extrapolated, cuts the Y-axis at 2.3. The intercept value is in sharp contrast to the unliganded tetramer, which has a $K_{sv}$ of 47 M$^{-1}$ and cuts the *Y*-axis at 4.5. The $O_R1$−repressor complex behaves very similarly to the unliganded dimeric repressor in this respect, which has a large amplitude of quenchable fluorescence (Bandyopadhyay *et al*., 1995). Although at 17 $\mu$M, greater than 50% of the operator−repressor complex is in tetrameric state, a significant amount of the repressor−operator complex is still in dimeric state. We have not attempted to do the experiment at higher protein concentrations due to the possibility of octamer and higher order aggregate formation. Similar $K_{sv}$ and intercept values for unliganded dimer and repressor−operator complex at 17 $\mu$M suggest no major change of W230 environment, notwithstanding the incompletion of the transition. In the unliganded tetramer, a large fraction of this quenchable fluorescence becomes nonquenchable, indicating the change of environment of a tryptophan which we had identified as W230 (Bandyopadhyay *et al*., 1995). The lack of such a change in the acrylamide quenchable fluorescence in $O_R1$−repressor tetramer complex suggests W230 remains exposed in the $O_R1$−repressor complex tetramer. Taken together with the thermodynamic data, this indicates that the operator-induced conformational change

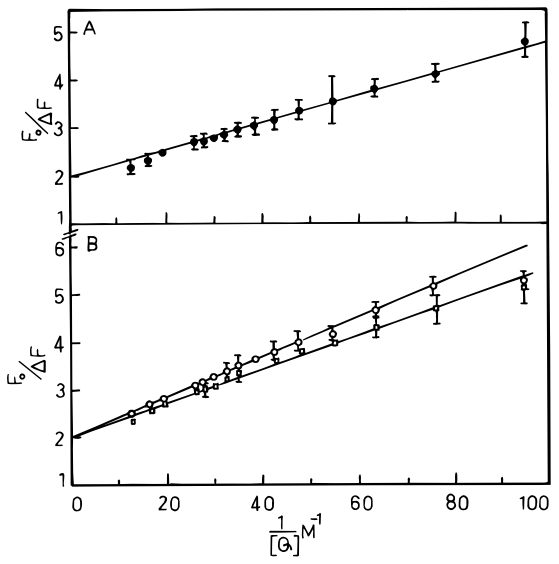


FIGURE 3: The Lehrer plot of acrylamide quenching of (A) 0.5 $\mu$M (●) λ-repressor and (B) 0.5 $\mu$M λ-repressor in presence of a stoichiometric amount of wild-type double-operator $O_R1-O_R2$ (□) and control double-operator $O_R1-O_R2$ (○). The solution conditions were 0.1 M phosphate buffer, pH 8.0, 25 °C. The excitation and emission wavelengths were 295 and 340 nm, respectively. Each point is an average of four independent determinations.

in the C-terminal domain causes significant change in the dimer−dimer interface.

To investigate if such a difference in the dimer−dimer interface also exists in the adjacent operator-bound tetramer complex, we have studied an oligomer (45 mer) of wild-type sequence containing $O_R1$ and $O_R2$. As a control, we have studied a sequence of same composition and length, in which the two operators are separated by four additional base pairs, i.e., approximately a half-turn of B-DNA. The separation between the pseudo-dyad axes of $O_R1$ and $O_R2$ in the sequence is 23 base pairs, that is, approximately two turns of B-DNA. If the two repressor dimers are approximately on the same side of DNA in the wild-type sequence, then they will be on the opposite face after the introduction of the four base pairs in between, thereby preventing cooperative interaction. Such effects have been observed in many DNA binding proteins, including the λ-repressor (Hochschild & Ptashne, 1986). Figure 3 shows the Lehrer plot of acrylamide quenching of the unliganded repressor, the wild-type $O_R1-O_R2$−repressor complex and the control $O_R1-O_R2$−repressor complex. The initial part of the free repressor plot cuts the *Y*-axis around 2, with $K_{sv}$ of 27 $M^{-1}$, consistent with our previous report. The initial part of the Lehrer plot, is very similar for all the operator complexes, cutting the *Y*-axis around 2. The most notable feature is the *Y*-axis intercept of around 2 for the wild-type $O_R1-O_R2$−repressor complex, in spite of the existence of dimer−dimer contact. This is in sharp contrast to the unliganded tetramer, which shows an *Y*-axis intercept of over 4, as stated before. This suggests that like the $O_R1$−repressor tetramer complex, protein contact in $O_R1-O_R2$−repressor complex does not lead to significant change in the environment of tryptophan 230.

The critical assumption in the above analysis is that the wild-type $O_R1-O_R2$−repressor complex indeed undergoes dimer−dimer contact under the solution conditions used in this study. One way to demonstrate such a contact may be to demonstrate the distortion of the intervening DNA sequences upon formation of contact between $O_R1$- and $O_R2$-

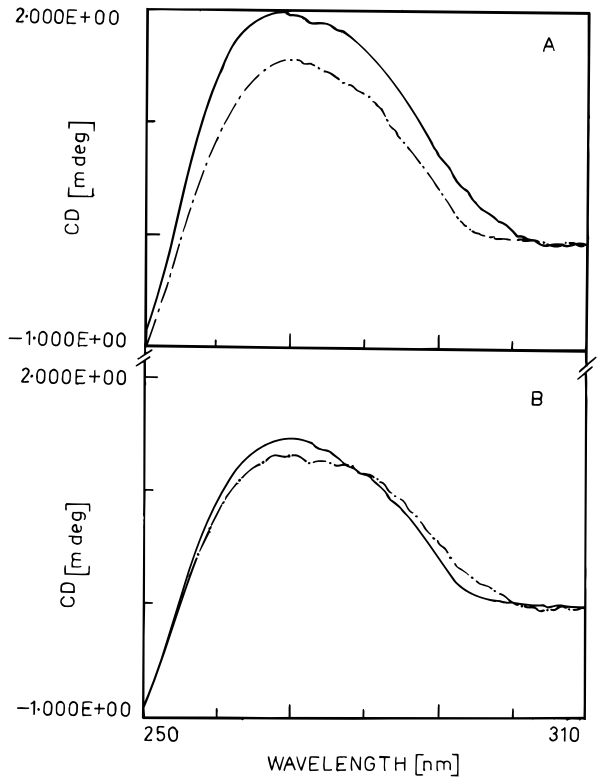


FIGURE 4: Circular dichroism spectra of (A) 0.25 $\mu$M wild-type $O_R1-O_R2$ (-·-) and wild-type $O_R1-O_R2$ in presence of 1.0 $\mu$M wild-type repressor (—) and (B) 0.25 $\mu$M control $O_R1-O_R2$ (-·-) and control $O_R1-O_R2$ in presence of 1.0 $\mu$M wild-type repressor (—) in 0.1 M phosphate buffer, pH 8.0, at ambient temperatures, i.e., approximately 25 °C. The spectra were taken in a 10 mm pathlength quartz cell.

bound repressors, as was recently reported by Strahs and Brenowitz (1994).

These distortions, characteristics of the repressors bound cooperatively to $O_R1$ and $O_R2$, may be reflected in CD spectra, as has been shown for several DNA binding proteins (Wartell & Adhya, 1988; Torigoe et al., 1991). In the 250−300 nm range, the protein CD spectra is much weaker than the nucleic acid spectra. Thus, changes reflected in the CD spectra must occur from nucleic acid conformational changes and not from that of the protein. Figure 4a shows the CD spectra of the wild-type $O_R1-O_R2$ sequence in solution and in the complex with two repressor dimers. The spectra are distinctly different in the 250−270 nm range, indicating DNA conformational difference in the complex when compared to the unliganded wild-type $O_R1-O_R2$. Figure 4b shows the CD spectra of the control $O_R1-O_R2$ in the unliganded and the liganded states. The spectra show only small differences, suggesting little additional distortion of the DNA upon the repressor binding. Since, the only difference between the wild-type and the control $O_R1-O_R2$ sequence is the cooperative contact, the change in CD spectra in the wild-type sequence is likely to be a reflection of DNA distortion that accompanies cooperative dimer−dimer contact. Although it is difficult to quantitate the distortion, it appears that a significant part of the DNA helix may be distorted in the complex. Strahs and Brenowitz (1994) have recently studied DNA distortion in the λ-repressor−$O_R$ complex and came to the conclusion that the DNA base pairs in between the two operators are distorted in the complex. This agrees well with the difference CD data shown above. This also indicates that under the conditions of the acrylamide quench-

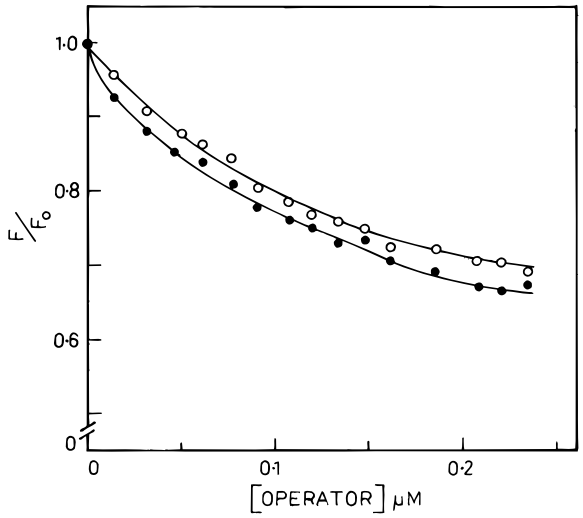


FIGURE 5: Plot of quenching of acrylodan fluorescence of 0.5 $\mu$M of mutant $\lambda$-repressor at 235C by addition of wild-type $O_R1-O_R2$ (●) and control $O_R1-O_R2$ (○). The solution conditions were 0.1 M phosphate buffer, pH 8.0, 25 °C. The excitation and emission wavelengths were 390 and 510 nm, respectively. The excitation and the emission band passes were set at 5 nm.

ing experiments the dimer−dimer contacts are taking place as expected.

If W230 environment is significantly different in the unliganded tetramer and the adjacent operator (wild-type) bound tetramer, then it is possible that the C-terminal tail region may participate in protein−protein contact in a different way when free in solution or when bound to adjacent operator sites. Such a conclusion is suggested also by the differential effect of S228N mutation on unliganded tetramer formation and cooperativity. Previously, we have shown that F235C $\lambda$-repressor labeled with acrylodan at C235 shows significant fluorescence enhancement and shifts of emission maximum, upon unliganded protein association in solution (unliganded) (Bandyopadhyay *et al*., 1995). Figure 5 shows the intensity of acrylodan fluorescence when 0.5 $\mu$M acrylodan labeled F235C $\lambda$-repressor is titrated with the wild-type $O_R1-O_R2$ fragment and the control $O_R1-O_R2$ fragment. Significant fluorescence quenching is seen in both the cases, the magnitude of which is only slightly higher in the wild-type $O_R1-O_R2$ fragment than in the control $O_R1-O_R2$ case. This indicates that upon operator binding, fluorescence quenching takes place, which may arise from the operator-induced conformational change described previously (Saha *et al*., 1992). Similar quenching is seen for single operator $O_R1$ (data not shown). After correcting for the effect due to single-operator binding, a small additional quenching may be present in the wild-type $O_R1-O_R2$, which may be attributed to protein−protein contact between the adjacent operator-bound dimers. The small additional fluorescence quenching, however, is qualitatively different from the association of the unliganded dimers, which shows large fluorescence enhancement. This definitely suggests that the environment of C235 is different in the unliganded and the adjacent operator (wild-type)-bound tetramers.

The association of $\lambda$-repressor in solution has been studied by several groups (Brack & Pirrotta, 1975; Chadwick *et al*., 1970; Laue *et al*., 1993). Although it has been generally accepted that both tetramers and octamers form at higher concentrations, the degree of coupling between the dimer−tetramer and the tetramer−octamer transitions has remained disputed. Laue *et al*. (1993) have reported strong coupling

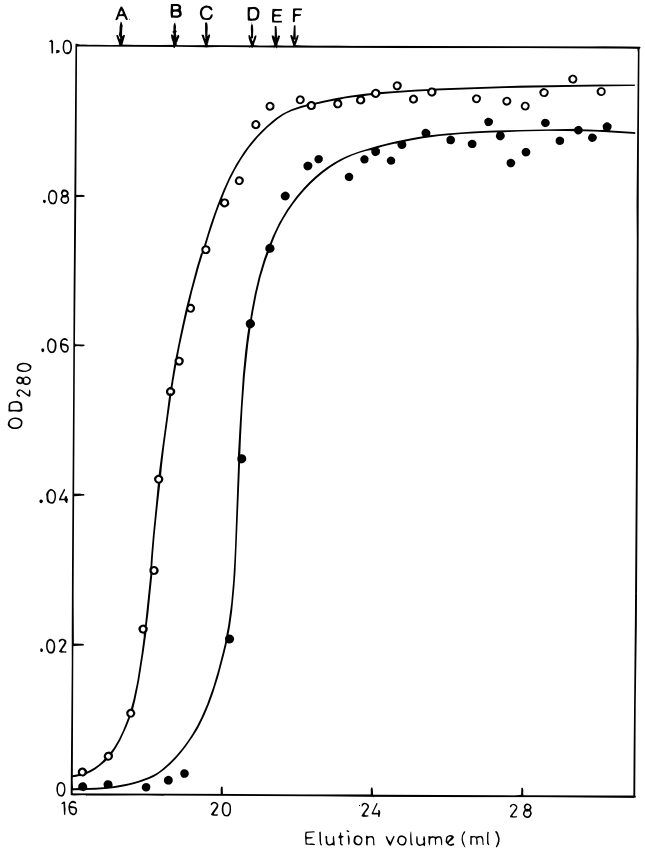


FIGURE 6: Elution profile of large-zone gel filtration of 15 $\mu$M $\lambda$-repressor, (●) in 0.1 M phosphate buffer, pH 8.0, and (○) in 10 mM tris-HCl, pH 8.0, containing 1 mM $CaCl_2$, 2.5 mM $MgCl_2$, 0.2 M KCl, and 1 mM $\beta$-ME. A, B, C, D, E, and F are the elution positions of appoferritin, $\beta$-amylase, yeast alcohol dehydrogenase, tubulin, bovine serum albumin, and carbonic anhydrase, respectively.

between the two transitions, whereas we have observed weak coupling, not inconsistent with the conclusions of previous studies (Banik *et al*., 1993). The major difference between the two studies is that the former contains millimolar concentrations of divalent cations, $Mg^{2+}$ and $Ca^{2+}$, whereas the latter studies were done in buffers devoid of divalent cations. Since it is important to the central point of this article that we are indeed observing unliganded tetramers and not octamers at concentrations studied here, we have re-investigated this problem. One of the major deficiencies in our previous paper (Banik *et al*., 1993) was that the techniques used in the molecular weight determination were all of nonequilibrium nature. To supplement that, we have chosen two techniques which can yield information on the state of aggregation under equilibrium conditions. Pioneering works by Ackers and co-workers (Ackers, 1975; Koblan & Ackers, 1991b; Burz *et al*., 1994) have demonstrated the use of large-zone gel filtration to study concentration dependent association equilibria in proteins. They have shown that under the large zone conditions, the weight average molecular weight of an associating system, at a particular protein concentration, may be obtained. Figure 6 shows large-zone gel filtration of $\lambda$-repressor at concentrations around 15 $\mu$M in 0.1 M potassoium phosphate, pH 8.0, and in 10 mM tris-HCl, pH 8.0, containing 0.2 M KCl, 1.0 mM $\beta$-ME, 1.0 mM $Ca^{2+}$, and 2.5 mM $Mg^{2+}$. The elution profile is significantly different, with $\lambda$-repressor in phosphate buffer eluting considerably later than $\lambda$-repressor in tris buffer containing divalent cations. The weight average molecular

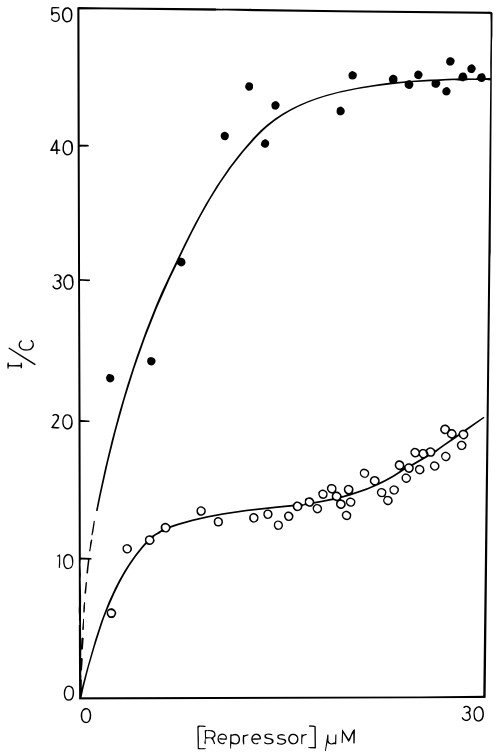


FIGURE 7: Plot of $I/[C]$ vs $[C]$ (○) in 0.1 M phosphate buffer, pH 8.0, (●) in 10 mM Tris−HCl buffer, pH 8.0, containing 1 mM $CaCl_2$, 2.5 mM $MgCl_2$, 0.2 M KCl, and 1 mM β-ME. Experiments were done at 25 °C. The excitation and emission wavelengths were 330 nm. The band passes were set at 5 nm.

weights measured from the centroid of the leading edge and from the plateau concentrations are significantly different. The column was calibrated using the following proteins: carbonic anhydrase, BSA, tubulin, yeast alcohol dehydrogenase, β-amylase, and apoferritin. The derived weight average molecular weight of λ-repressor in phosphate buffer at 15 μM protein concentration is 79 500 and in the tris buffer containing divalent cations is 209 000 at 15.4 μM protein concentration. This suggests that at around 15 μM, in the presence of divalent cations, the octamer may be the predominant species, whereas in the absence of divalent cation, very little octamer may be present.

Light scattering may be used to study protein association at equilibrium, since at first approximation, at a given protein concentration, the scattering intensity is proportional to the weight average molecular weight of the protein ($I = KMC$, where $I$ is the scattering intensity, $C$ is the protein concentration, $M$ is the weight average molecular weight and $K$ is a constant). Thus, the scattered intensity/protein concentration versus protein concentration ($I/C$ vs $C$) plot should be linear and parallel to the concentration axis for nonassociating systems. We have measured relative scattering intensities in a fluorimeter to establish an association profile rather than the absolute molecular weights. Figure 7 shows the light-scattering intensity of λ-repressor at different concentrations in two different buffers as previously mentioned. The scattering intensity versus protein concentration profile is very different in the two buffers. In tris buffer containing divalent cations, the intensity increases very rapidly and then flattens out before 15 μM. In the phosphate buffer, the scattered intensity increases relatively slowly and reaches less than half the scattering intensity as that in the tris/divalent cation buffer at around 15 μM. At higher protein concentrations, there is still a further increase of scattering intensity. Since the scattering intensity at a given concentration is proportional to the weight average molecular weight of the species, it is likely that at around 15−20 μM protein concentration the repressor is tetrameric and the octamer formation occurs above 20 μM. This agrees well with the large-zone gel filtration data reported above. To verify that the scattering intensities in the two buffers are indeed different, we have directly Millipore filtered (syringe filters, 0.45 μm) protein solutions in the two above mentioned buffers at approximately 20 μM concentrations into a cuvette and measured the scattering intensity. The scattering intensity in the tris/divalent cation buffer is approximately twice that in the phosphate buffer (data not shown), confirming the observations made above.

## DISCUSSION

The regulation of the right operator of the bacteriophage λ, has been investigated extensively. The homologous and the heterologous protein−protein contacts play a crucial role in regulating this genetic switch. The homologous λ-repressor contacts are all believed to take place through the C-terminal domain of λ-repressor. The lack of a crystal or NMR structure, however, has hampered the understanding of the structural and molecular basis of cooperative binding to operator sites.

Recently, a large number of non-cooperative mutants have been isolated (Benson *et al*., 1993; Bain & Ackers 1994). They map into several regions of the repressor. It appears that the region 188−212 may be the crucial region in terms of cooperative contact. There are also other regions that may be involved, e.g., 147 (Beckett *et al*., 1993) and the C-terminal tail region (Benson *et al*., 1994; Whipple *et al*., 1994). λ-Repressor also undergoes association in solution to tetramers and higher order structures (Brack & Pirota, 1975). The nature of this transition has been the subject of dispute in recent literature. Although several earlier reports (Brack & Pirrotta, 1975; Chadwick *et al*., 1970) and one recent report (Banik *et al*., 1993) suggest that dimer to tetramer and tetramer to octamer transitions are weakly coupled, a recent report suggests that it is strongly coupled (Laue *et al*., 1993). The solution conditions in these studies, however, differed significantly, particularly with respect to the presence of divalent metal cations. The nature of the protein−protein contact under different conditions have, however, remained unclear.

One of the most interesting mutant studies has been reported by Burz and Ackers (1994). They have characterized aggregation properties of several non-cooperative mutants including S228N. In contrast to the characterization of S228N by Hochschild and Ptashne (1988) as a non-cooperative mutant, Burz and Ackers (1994) have reported that the mutant is defective in monomer−dimer association but retains full degree of protein−protein interactions responsible for DNA binding cooperativity. Interestingly, this mutant is defective in aggregation to higher order forms in solution. This suggests that the protein−protein interface in unliganded tetramers and adjacent operator bound tetramers may be different. Hence we have examined this very interesting question by spectroscopic techniques and structural analysis.

Crucial to the comparison of dimer−dimer interface is the unambiguous demonstration that in the unliganded state the tetramers are the predominant species at intermediate con-

centrations. We have used two equilibrium techniques, large-zone gel filtration and light scattering, to demonstrate that under the conditions of our study, the weight average molecular weight of the species present is indeed indicative of the presence of a predominant amount of tetramers. The light-scattering profile indicates weak coupling between dimer−tetramer and tetramer−octamer transitions under our solution conditions and strong coupling under the conditions reported by Ross *et al*. (1993). These data taken together with data reported by previous studies (Banik *et al*., 1993) indicate that solution conditions, possibly divalent metal cations, may strongly influence the degree of coupling between dimer−tetramer and tetramer−octamer transitions and it is possible to observe unliganded tetramers predominantly in solution.

Previously we have shown by fluorescence quenching and by the use of an external covalent probe that the C-terminal tail region of the $\lambda$-repressor may be involved in the tetramerization in the unliganded state in solution (Bandyopadhyay et al., 1995). Both W230 and Acrylodan-C235 probes showed significant additional shielding from solvent upon unliganded tetramer formation. The environments of these probes, however, are unaffected by dimer−dimer contact, when the repressor is bound to adjacent operators. This taken with the S228N reported by Burz and Ackers (1994) strongly suggests that the C-terminal tail region is involved in the tetramer formation in unliganded situations but not in the adjacent operator-bound case. Several other non-cooperative mutants, e.g., Gly147-Asp, Lys192-Asn, and Tyr210-His, however, are defective in aggregation in the unliganded state (Burz & Ackers, 1994). This suggests that part of the dimer−dimer interface may be common and the major difference may lie in the role of the C-terminal tail region.

This raises an interesting question, that of whether the difference in the dimer−dimer contact is due to binding to operator, i.e., the difference is caused by the operator-induced conformational change reported previously (Saha *et al*., 1992), or is due to spatial constraint of being bound to adjacent operator sites. Previously, it has been reported that the unliganded repressor association is strongly enthalpy driven (Banik *et al*., 1993), whereas the cooperative interaction energy derived from quantitative footprinting experiments (Senear *et al*., 1986) has an enthalpy of near zero. This prompted us to examine the thermodynamics of association of the single-operator-bound repressor dimers and the environment of W230 in tetramers bound to two isolated single operators. The thermodynamics of single-operator-bound repressor dimers are strongly entropy driven with near-zero enthalpy. The fluorescence-quenching studies indicate that the environment of W230 is like that of unliganded dimers. This shows that the difference in interface, between unliganded tetramers and adjacent operator-bound tetramers, may predominantly be due to operator binding and consequent conformational change. A large degree of quenching of acrylodan fluorescence of F235C-labeled acrylodan, upon operator binding, suggests a conformational change involving the C-terminal tail region. This may cause the role of the C-terminal tail region in the protein−protein contact to change.

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