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A Fluorescence Anisotropy Study of Tetramer-Dimer Equilibrium of λ Repressor and Its Implication for Function*

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Tetramer-dimer equilibrium of λ repressor has been studied by fluorescence anisotropy techniques. We have chosen 1-dimethylamino napthalene-5-sulfonyl chloride (dansyl chloride)-labeled repressor to study the dissociation-association equilibrium, because of relatively long life-time of the probe (>10 ns). Polarization of the dansyl-labeled repressor decreases with decreasing protein concentrations in the range of 20 to 0.2 μM. The decrease of anisotropy was shown to be due to reversible dissociation of the protein. Size exclusion high-performance liquid chromatography studies and polyacrylamide gel electrophoresis under native conditions (Ferguson plot) confirmed that at around 20 μM concentrations the repressor exists in predominantly tetrameric form, whereas in lower concentrations it exists in predominantly dimer form. A dissociation constant of 2.3 ± 0.9 μM was estimated in 0.1 M potassium phosphate, pH 8.0, at 25 °C. A stoichiometric amount of isolated single operator shifted the tetramer-dimer equilibrium toward the dimer. Increased ionic strength had only a modest effect on the dissociation constant. The thermodynamic constants for the dissociation reaction calculated from the Van't Hoff plot was +26.6 kcal/mol for ΔH and +64.7 e.u. for ΔS. The rotational correlation times derived from isothermal Perrin plot indicated elongated dimers and tetramers.

λ repressor is a DNA binding protein with multiple functions. It binds selectively to specific operator sequences in the bacteriophage λ genome. The binding to adjacent operators are characterized by positive co-operativity (Senear *et al.*, 1986). In addition, it also functions as an activator of transcription (Ptashne, 1985). At submicromolar concentrations it exists as a dimer of two identical subunits (Brack and Pirotta, 1975; Chadwick *et al.*, 1970). This dimeric form binds to a single operator, and contacts between adjacent operator bound dimers are responsible for the positive co-operativity (Hochschild and Ptashne, 1986). The interaction energy between the two adjacent operator-bound repressors and related thermodynamic parameters are known from the quantitative DNase footprinting experiments (Senear *et al.*, 1986). This interaction energy is the sum of several effects, *e.g.* protein dimer-dimer interaction, proximity effects of two operator-bound repressor molecules, DNA distortion energy, etc. Understanding of co-operativity at the molecular level would require separation and study of these individual effects.

It is well established that λ repressor is a two domain protein. The N-terminal domain is responsible for DNA binding, and the C-terminal domain is responsible for dimerization as well as higher order contacts (Pabo *et al.*, 1979). To date, tetramer-dimer equilibrium has only been studied by ultracentrifugation (Brack and Pirotta, 1975; Chadwick *et al.*, 1970), and a tetramer-dimer dissociation range of 10^{-7} to 10^{-5} M has been obtained, although no precise dissociation constant was given. In the same article by Brack and Pirotta (1975), electron microscopy was used to arrive at some size and shape information of the dimer and the tetramer. Very little is known about the nature and energetics of dimer-dimer interaction or about the influence of operator binding on the tetramer-dimer equilibrium.

Due to the obvious importance of dimer-dimer association in the functioning of λ repressor, we have chosen to study the tetramer-dimer equilibrium by fluorescence anisotropy techniques. Subunit dissociation can be effectively monitored by fluorescence anisotropy (Shore and Chakravarti, 1976) provided the dissociated species have substantially different anisotropy from the associated species. Internal tryptophan residues are generally unsuitable to monitor dissociation because of short fluorescence lifetimes. An external probe of longer lifetime would be desirable to monitor tetramer-dimer dissociation in λ repressor. Though the λ repressor has 3 cysteine residues (Sauer and Anderegg, 1979), they are all unreactive under native conditions and hence are unsuitable for attaching fluorescent probes (Banik *et al.*, 1992). Reaction with fluorescein isothiocyanate causes modification of the N-terminal arm which is highly mobile, resulting in very low anisotropy values. This situation is undesirable for following the tetramer dissociation. Dansyl chloride[1]-labeled protein, however, gave high anisotropy values indicating rigidness of the attached probe and hence its suitability as a probe for tetramer-dimer dissociation. In addition, dansyl chloride has a fluorescence lifetime of approximately 10 ns or more, which would be suitable for monitoring dissociation of a 100,000-dalton protein to 2 × 50,000-dalton proteins. In this article, we have used dansyl chloride-labeled λ repressor to study various aspects of its tetramer-dimer equilibrium.

EXPERIMENTAL PROCEDURES

Materials—Dansyl chloride, sucrose, ovalbumin, bovine serum albumin, yeast alcohol dehydrogenase, blue dextran, lysozyme, cytochrome *c*, apoferritin, and β-amylase were obtained from Sigma. Sephadex G-50 was purchased from Pharmacia United (Sweden). Goat brain tubulin was prepared according to Mukhopadhaya *et al.* (1990) (see references therein). Glutaminyl-tRNA synthetase from

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[1] The abbreviations and trivial name used are: dansyl chloride, 1-dimethylamino napthalene-5-sulfonyl chloride; bp, base pair(s); HPLC, high-performance liquid chromatography.

Escherichia coli was prepared according to Bhattacharyya *et al.* (1991). All other reagents were of analytical grade. Plasmid pEA305 was a gift from Prof. Mark Ptashne. Crude 17-bp O_R1 was a gift from Prof. J. S. Cohen and was further purified according to Stec *et al.* (1985) in a LKB HPLC using a reversed-phase C_{18} column.

Repressor Isolation—λ repressor was isolated from *E. coli* RR1 15 ΔlacZ carrying a plasmid pEA305 which contains wild type cI gene under the control of tac promoter (Amann *et al.*, 1983). The cells containing the above plasmid were grown up to OD_{550} = 0.6, then IPTG at a final concentration of 1 mM was added and grown for a further 2 h. Repressor was purified according to the method I of Johnson *et al.* (1980). λ repressor was assayed by filter binding procedure of Riggs *et al.* (1970). The purified repressor showed a single band of molecular weight of 26,000 on SDS-PAGE (Laemmli, 1970). Protein was estimated by absorbance measurement at 280 nm using a $E_{280}^{1\%}$ value of 11.3. The repressor concentration was always calculated in terms of monomer unless stated otherwise. For all studies, the repressor was dialyzed against 0.1 M potassium phosphate buffer, pH 8.0, unless specifically mentioned otherwise.

Chemical Modification—The repressor was dansyl-labeled by treatment with a 10-fold molar excess of dansyl chloride. A DMF solution of dansyl chloride was added to a 0.5 mg/ml solution of λ repressor in 0.24 M potassium phosphate buffer, pH 8.0, so that the final DMF concentration was 5% in the reaction mixture. The reaction was performed at 25 °C for 15 min, quenched with 1 M Tris-HCl, pH 7.8, to a final concentration of 5 mM Tris-HCl, and loaded onto a Sephadex G-50 column (1.0 × 15 cm) equilibrated with 0.1 M potassium phosphate buffer, pH 8.0. The appropriate fractions were then pooled and dialyzed overnight against 0.1 M potassium phosphate buffer, pH 8.0. Labeled repressor concentration was determined by Bio-Rad protein assay (Bradford, 1976) using purified λ repressor as standard. The incorporation ratio of dansyl-labeled repressor was calculated to be 1.1 ± 0.2 using a molar extinction coefficient of 4500 M^{-1} cm^{-1} at 340 nm (Matthews and Hsieh, 1985). This value was the average of 10 determinations. Activity measurements of dansylated λ repressor was done by nitrocellulose filter binding assay according to Riggs *et al.* (1970).

Fluorescence Studies—Steady state fluorescence was measured in a Hitachi F 3000 spectrofluorometer equipped with a computer for spectra addition and subtraction. The bandpass of both excitation and emission monochromators were 5 nm unless stated otherwise. All fluorescence measurements were done at 25 °C, unless specifically mentioned otherwise. The experiments were carried out in 1-cm path length cuvettes. Anisotropy experiments were performed using Hitachi polarization accessories. The fluorescence intensity components (I_{vv}, I_{vh}, I_{hv}, I_{hh}), in which the subscripts refer to the horizontal (h) or the vertical (v) positioning of the excitation and emission polarizers, respectively, were used to calculate the steady-state fluorescence anisotropy (A) as follows.

$$A = \frac{I_{vv} - GI_{vh}}{I_{vv} + 2GI_{vh}} \qquad \text{(Eq. 1)}$$

Where G is the grating factor that corrects for wavelength dependent distortion of the polarizing system as follows.

$$G = \frac{I_{hv}}{I_{hh}} \qquad \text{(Eq. 2)}$$

In a typical experiment, to obtain tetramer-dimer dissociation constant, dansyl-labeled repressor was mixed with unlabeled repressor at a ratio of 1:9 to achieve a high concentration (approximately 20 μM). The samples were then progressively diluted to various concentrations with 0.1 M potassium phosphate buffer, pH 8.0, except in the experiments where the effect of salt on tetramer dissociation was observed. In the latter case, dilution was carried out by the addition of 0.1 M potassium phosphate, pH 8.0, containing appropriate concentrations of NaCl. In case of determination of the effect of operator on dissociation, a 1:1 complex of repressor dimer to O_R1 was progressively diluted with buffer so as to maintain the 1:1 ratio. In all cases, buffer blank value was subtracted from each experimental value. To increase the signal-to-noise ratio, computer averaging was performed when needed. Energy transfer efficiencies were calculated as given in Saha *et al.* (1992).

To determine the correlation time and limiting anisotrophy, an isothermal Perrin plot was done. The Perrin equation is given below.

$$1/A = 1/A_o[1 + \tau_f/\tau_c] = 1/A_o[1 + \tau_f kT/V_h\eta) \qquad \text{(Eq. 3)}$$

In this equation, τ_f is the fluorescence life-time, τ_c is the rotational correlation time, k is the Boltzmann constant, T is the temperature, V_h is the molecular volume and η is the viscosity of the solution. Viscosity was varied at constant temperature by the inclusion of sucrose in 0.1 M potassium phosphate buffer, pH 8.0. Literature values for the viscosity of sucrose were used (Weast, 1977).

Fluorescence lifetimes were determined in an Applied Photophysics single photon counting apparatus using a nitrogen lamp for excitation. The excitation wavelength was 337 nm, and the emission wavelength was 520 nm. The wavelength selection was done by monochromators. The decay curves were fitted to single and biexponential functions after deconvoluting the lamp response function. The lifetime of dansyl-labeled repressor was determined at 25 °C in 0.1 M potassium phosphate buffer, pH 8.0, at protein concentrations of 0.5 μM, where the repressor is mostly dimer and 20 μM, where the repressor is mostly tetrameric.

Size Exclusion HPLC and Ferguson Plot—Size exclusion HPLC was done in a Waters HPLC using a protein Pak™ 300SW column (7.8 × 300 mm, fractionation range 10,000–400,000 daltons). The column was pre-equilibrated and eluted with 0.1 M potassium phosphate buffer, pH 8.0, and a 0.4 ml/min flow rate was used throughout. Ovalbumin, bovine serum albumin, tubulin, β-amylase, apoferritin, and glutaminyl-tRNA synthetase were used as molecular weight markers. Blue dextran was used to determine the void volume and tryptophan and cytochrome *c* were used to obtain the column volume.

Polyacrylamide gel electrophoresis was conducted in a continuous buffer system (0.1 M Tris-HCl, pH 8.0) at room temperature. 3, 4, 5, and 6% gels were polymerized in different tubes, so that the gel lengths were identical. The samples were applied at a concentration of 20 μM, except tubulin, which was applied at 5 μM. All the samples contained 10% glycerol and the tracking dye bromphenol blue (0.01%). The gels were run at a constant voltage of 100 V. After the completion of the run the length of the gel and the length of migration of the dye was measured. The length of the gel and the length of the migration of the protein were measured after staining and destaining. The relative mobility of the protein with respect to the dye is the ratio of migration of the dye and the protein after correction for the expansion of the gel. The slopes (K_R) of the log (R_f) *versus* %T plots were obtained from the best fit lines, where R_f refers to relative mobility and %T refers to gel concentration. The molecular weight of λ repressor was calculated from a plot of $K_R^{1/2}$ *versus* molecular radius, which was constructed from four standard proteins (apoferritin, β-amylase, tubulin, BSA) (Rodbard and Chrambach, 1969).

Simulation of Dimer-Tetramer Equilibrium—Mathematical expression of observed anisotropy as a function of total protein concentration was obtained by combining Weber average formula (Weber, 1952) with the expression for tetramer-dimer equilibrium constant as follows.

$$A = \frac{K_d \cdot [-1 + \sqrt{(1 + 4P_o/K_d)}] \cdot \{2A_d + A_t[-1 + \sqrt{(1 + 4P_o/K_d)}]\}}{4P_o} \qquad \text{(Eq. 4)}$$

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, tetramer anisotropy, and the dimer anisotropy. The three unknown parameters are systematically varied within a given range and the χ^2 statistic was used to judge the quality of fit with the experimental data (a complete grid search). The set of parameter values that gave the minimum χ^2 value, was chosen as the best-fit curve.

RESULTS

Characterization of Dansyl-labeled Repressor—After gel filtration and dialysis, incorporation was 1.1 ± 0.2 mol of dansyl per mol of repressor monomer. The DNA binding activity of the dansylated repressor was measured by titration of [λ-^{3}H] DNA using nitrocellulose filter binding assay (Riggs *et al.*, 1970). The protein concentration at which half-maximal saturation was reached, was obtained from titration curves with dansylated λ repressor and unmodified repressor. The titration curves and midpoints of titration was found to be almost

identical for both the modified and unmodified repressor (data not shown). This indicates that the dansylated λ repressor retains full DNA binding activity.

Fig. 1 shows the difference absorption spectra and the excitation spectra of the dansylated repressor. If there is no energy transfer, the absorption spectra of the dansyl group should match the excitation spectra. The higher intensity of the excitation spectra, compared to the absorption spectra, in the region of 295 nm indicated energy transfer from the tryptophan residues of the protein. Calculated energy transfer efficiency (E) (Cantor and Schimmel, 1980) is very high (E = 175%), indicating a high degree of energy transfer from more than one tryptophan. Such high degree of energy transfer indicates that the dansyls are close to the tryptophans. Since all three tryptophans in the repressor are in or near the C-terminal domain, the modifications are likely to be in or near the C-terminal domain.

The fluorescence lifetimes of dansylated repressor was determined at 0.5 and 20 μM concentrations. The real time decays of the dansyl fluorescence are shown in Fig. 2. Dansyl fluorescence decays are single exponentials in some cases and biexponential in others (Williams *et al.*, 1982; Royer *et al.*, 1990). We have fitted the fluorescence decay of the dansylated repressor to both bi- and single-exponentials. The quality of fit to a single exponential was good and fit to a biexponential did not significantly improve the reduced χ^2 values. The lifetime at high and low protein concentrations does not change significantly, indicating that the dansyl group would be suitable for studying tetramer-dimer dissociation (Table I).

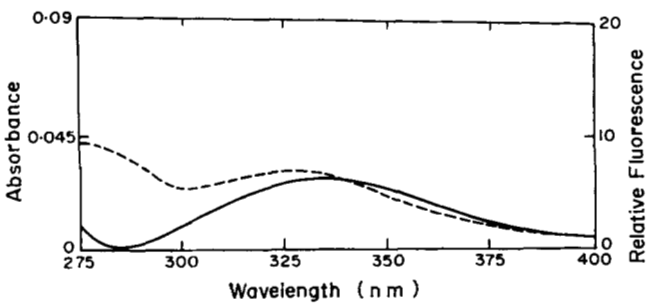

FIG. 1. **Fluorescence energy transfer between dansyl and tryptophans.** *Dotted line*, excitation spectra of the dansylated λ repressor. Emission wavelength was 500 nm. *Solid line*, difference absorption spectra of 7 μM dansyl repressor and 7 μM of unlabeled repressor. Both spectra were taken in 0.1 M potassium phosphate buffer, pH 8.0.

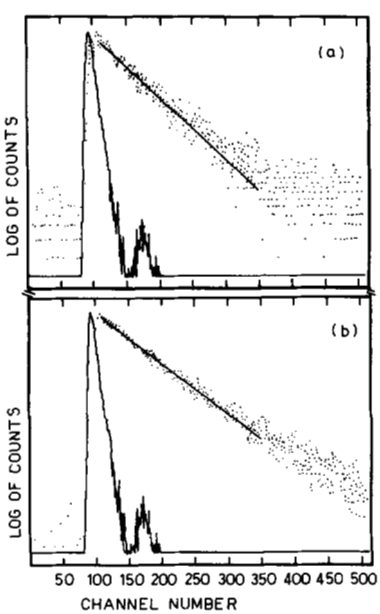

FIG. 2. **Lifetime measurement of dansylated λ repressor.** The excitation wavelength was 520 nm. A nitrogen lamp was used for excitation, and monochromators were used to choose excitation and emission wavelength. A low wavelength cut-off filter was used to cut off scattered lights. Each channel in the figure is 0.166 ns. *a*, λ repressor at 0.5 μM; *b*, λ repressor at 20.0 μM.

Molecular Weight Determination—Molecular weight of the repressor was determined by size exclusion HPLC in the concentration range where the anisotropy studies were carried out. Fig. 3 shows the elution profile of the repressor at three different protein concentrations. The inset shows the log molecular weight *versus* K_{av} value for several standard proteins. The elution position of the repressor at high and low protein concentrations are indicated by two *arrows*. The repressor when injected at high protein concentration (4.8 mg/ml) elutes as molecular weight of 100,000, and the repressor when injected at low protein concentration (0.5 mg/ml) elutes as a species of molecular weight 42,000. The peak concentration eluted was approximately 15 μM when the repressor was injected at high concentrations and approximately 1 μM when injected at low concentrations. This indicates that the λ repressor is predominantly a tetramer at higher concentrations and dimer at lower concentrations. The elution volume of λ repressor was studied as a function of loading concentration. At higher loading concentrations, the elution volume levels off to a value corresponding to a molecular weight of 100,000 daltons (data not shown). In small zone size exclusion experiments with dissociating systems undergoing fast exchange, elution volume has no simple relationship to molec-

TABLE I

Lifetimes and correlation times of λ repressor

λ repressor	Hydrated volume	τ_c	τ_f	Reduced χ^2 for τ_f
μM	$Å^3$	ns		
1.0	1.06×10^6	25	11.8	1.27
20.0	6.3×10^6	160	14.5	1.21

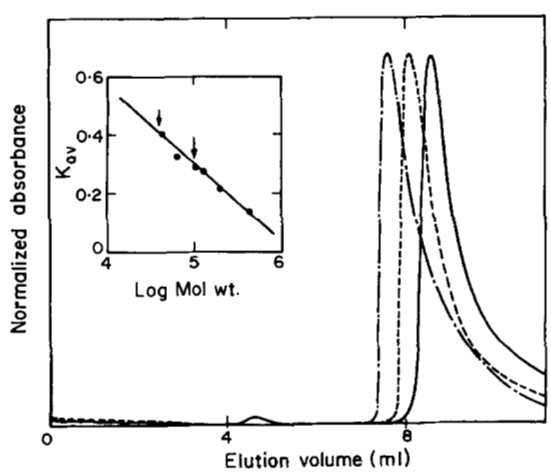

FIG. 3. **Effect of dilution on the elution behavior of λ repressor in size exclusion HPLC.** λ repressor at concentrations 4.8 mg/ml (–·–·–·–), 2.0 mg/ml (- - - - - -), and 0.5 mg/ml (——) was injected onto a Protein Pak™ 300 SW column. The column was pre-equilibrated with 0.1 M potassium phosphate buffer, pH 8.0. The flow rate was 0.4 ml/min. The inset (*a*) shows the K_{av} *versus* log molecular weight plot of several standard proteins. The *arrows* indicate elution position of λ repressor when injected at concentrations of 4.8 mg/ml (*right arrow*) and 0.5 mg/ml (*left arrow*).

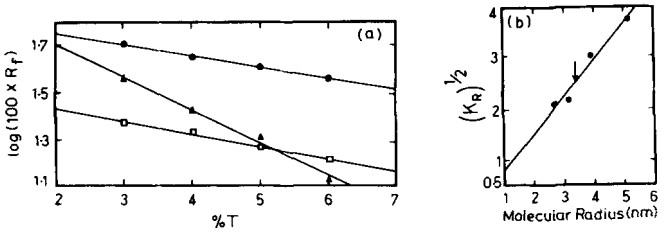

FIG. 4. **Molecular weight determination of λ repressor by polyacrylamide gel electrophoresis under native condition (Ferguson plot).** *a*, log (R_f × 100) *versus* %*T* plot for two standard proteins, apoferritin (▲), tubulin (●), and λ repressor (□). *b*, $K_R^{1/2}$ *versus* molecular radius for four standard proteins; apoferritin, β-amylase, tubulin, and bovine serum albumin. The *arrow* represents the position of λ repressor.

ular weights at either the concentrations loaded or peak elution concentrations. However, the leveling off of elution volume, corresponding to a molecular weight of tetramer, at higher loading concentrations suggests that the tetramer may be the limiting species present under this condition. As a control we have chromatographed a known monomeric protein ovalbumin at high and low concentrations. Ovalbumin elutes at identical position when injected at high and low concentrations. Our recent light-scattering experiments also suggest that tetramer may be the limiting species below 20 μM λ repressor concentration.[2]

Fig. 4 shows the log (R_f × 100) *versus* %*T* plot for λ repressor and two different standard proteins, apoferritin and tubulin. The inset shows the $K_R^{1/2}$ *vs.* molecular radius plot for four standard proteins (Rodbard and Chrambach, 1969). The *arrow* indicates the position of the λ repressor. The calculated molecular radius of λ repressor corresponds to a molecular weight of 130,000 (*i.e.* if it were an ideal sphere). The molecular weight of λ repressor at concentrations around 20 μM, as derived from size exclusion chromatography, native gel electrophoresis, as well as conclusions drawn previously from sedimentation velocity studies (Chadwick *et al.*, 1969) indicate that it is predominantly tetrameric under these conditions.

Fluorescence Anisotropy Studies—The anisotropy of the dansylated repressor was determined at various concentrations in the range of 20 to 0.2 μM. The anisotropy values decreased upon dilution. As a control, 5 μM dansylated λ repressor was diluted with 5 μM unlabeled repressor in 0.1 M potassium phosphate buffer, pH 8.0. No significant change of anisotropy was observed under this condition (data not shown). A plot of anisotropy values *versus* log repressor concentration produced a sigmoidal curve. Fig. 5 shows four such curves at 25 and 37 °C, with operator and in 0.6 M NaCl. Typical limiting anisotropy values obtained from the best fit are 0.26 and 0.14 for tetramer and dimer, respectively, at 25 °C. The obtained dissociation constant at 25 °C without operator was 2.3 ± 0.9 μM. This value is consistent with the range of tetramer-dimer dissociation obtained from ultracentrifugation studies (Brack and Pirotta, 1975; Chadwick *et al.*, 1970) and not too far from the corresponding Δ*G* value quoted in Hochschild and Ptashne (1986). In one experiment, reversibility of the dissociation was shown by first diluting the protein from 25 to 1 μM and adding unlabeled repressor to make a total protein concentration of 25 μM. The anisotropy value which was 0.17 at the start of dilution reached 0.13 at 1 μM. Upon addition of unlabeled repressor, the anisotropy value returned to 0.16, indicating reversibility of the dissociation process.

As pointed out previously, contact between two adjacent or

[2] U. Banik and S. Roy, unpublished observations.

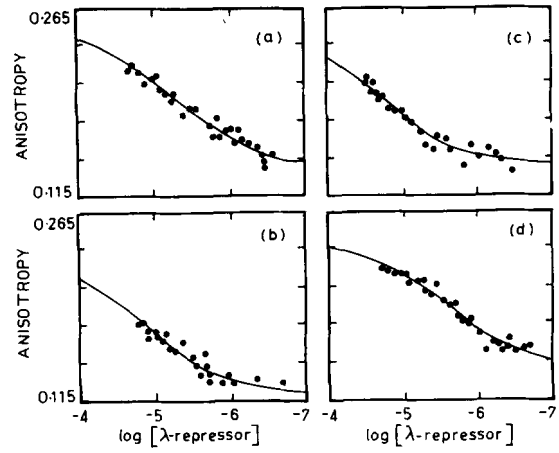

FIG. 5. **Tetramer-dimer dissociation curves at 25 °C (*a*), with operator O_R1 (*b*), and 37 °C (*c*), and 0.6 M NaCl (*d*).** The solution conditions were 0.1 M phosphate buffer, pH 8.0. The *curves* are the best fit curves as described under "Experimental Procedures."

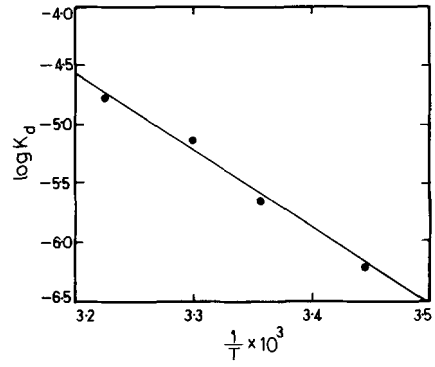

FIG. 6. **Van't Hoff plot for tetramer-dimer dissociation of λ repressor.** The dissociation constants at four different temperatures, 15, 25, 30, and 37 °C were determined as described under "Experimental Procedures." The solution conditions were 0.1 M potassium phosphate buffer, pH 8.0.

nonadjacent operator-bound repressor dimers are responsible for the co-operativity of repressor binding. Thus, to look into the energetics of the operator-bound dimer-dimer interaction, *i.e.* tetramerization, we have looked into the tetramer-dimer dissociation of a 1:1 complex of isolated single operator O_R1 to repressor dimer. As shown in Fig. 5*b*, the nature of the dissociation curve in the presence of operator is very similar, but has a dissociation constant of 8.3 ± 2.5 μM. Binding affinity of the repressor for the operator is so great that dilution in this protein concentration range is not likely to effect any significant degree of repressor dissociation from the operator (Johnson *et al.*, 1980). The operator binding thus weakens the dimer-dimer interaction significantly.

Thermodynamic Parameters of Dimer-Dimer Interaction—The Van't Hoff enthalpy and entropy was obtained from a Van't Hoff plot for tetramer-dimer dissociation constants at four different temperatures. Fig. 6 shows the Van't Hoff plot for tetramer-dimer dissociation, Δ*H* value of +26.6 kcal/mol and Δ*S* value of +64.7 e.u. was obtained for the tetramer-dimer dissociation. Thus the dimer-dimer association is an enthalpy-driven process, which is characteristic of some protein-protein associations (Klotz *et al.*, 1975). The magnitude of enthalpy and entropy is consistent with that of many proteins undergoing association. As shown in Table II, increased ionic strength has only a modest effect on tetramerization. Such large enthalpic contribution often results from Van der Waals interactions and hydrogen bonds (Ross and

TABLE II

Tetramer-dimer dissociation constants of λ repressor under various conditions

Solution conditions[a]	K_d	χ^2
	μM	
15°C	0.65 ± 0.35	1.3×10^{-2}
25°C	2.3 ± 0.9	1.6×10^{-2}
30°C	8.1 ± 2.1	1.6×10^{-2}
37°C	17.9 ± 1.2	8.6×10^{-3}
+0.6 M NaCl	1.0 ± 0.6	3.8×10^{-3}
+1.2 M NaCl	0.6	7.7×10^{-3}
+Stoichiometric amount of O_R1[b]	8.3 ± 2.5	1.3×10^{-2}
In Tris-HCl[c]	4.0	1.1×10^{-2}

[a] All experiments were conducted in 0.1 M potassium phosphate, pH 8.0 at 25°C. Additions and alterations are specifically indicated in the table.

[b] Repressor dimer: O_R1 = 1:1.

[c] The experiment was done in 0.1 M Tris-HCl, pH 8.0.

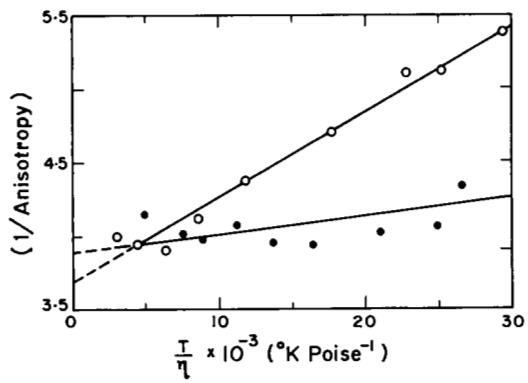

FIG. 7. **Perrin plot of dansylated λ repressor at two different repressor concentrations.** Total protein concentrations were (○) 1 and (●) 20 μM. Viscosity was varied isothermally (at 25 °C) by the inclusion of sucrose in 0.1 M potassium phosphate, pH 8.0.

Subramanian, 1981). Weak ionic strength dependence also suggests that ionic interactions do not play a very significant role in association of λ repressor dimers.

Size and Shape of the Dimer and Tetramer—To obtain size and shape information on dimeric and tetrameric λ repressor, we have calculated limiting anisotropy and rotational correlation time from the Perrin plot. The correlation times and lifetimes of λ repressor are summarized in Table I. The viscosity of the media was varied under isothermal conditions by inclusion of sucrose in the solution. Fig. 7 shows the Perrin plot of dansylated λ repressor under conditions where the repressor is in predominantly dimeric or tetrameric form. The limiting anisotropies in both cases are high and approximately equal (0.25). This is substantially less than the theoretical limiting anisotropy. It is well known that in isothermal Perrin plots theoretical limiting anisotropy is rarely reached and a value of 0.2 is quite common (Yguerabide, 1972). This indicates only minimal local motion of the probe. The hydrated volumes and rotational correlation times were derived from the value of the slope and fluorescence lifetimes (see Table I). The rotational correlation time of the dimer was found to be 25 ns. A perfectly spherical molecule of 52 kDa is expected to have a rotational correlation time of 21.5 ns (Cantor and Schimmel, 1980). The increased rotational correlation time is generally attributed to elongated molecular shapes. The ratio of the rotational correlation times, 25/21.5 = 1.15, is a function of the axial ratio of the ellipsoid that approximates the molecule (Benecky *et al.*, 1990). A ratio of 1.15 is consistent with either a prolate ellipsoid of axial ratio of 2:1 or an oblate ellipsoid of axial ratio of 1.4:1. The repressor tetramer was found to have a rotational correlation time of 160 ns, as compared to an expected correlation time of 43 ns for a perfect sphere. The ratio 160/43 = 3.7 is too large for a prolate ellipsoid and corresponds to an axial ratio of 7.5:1 for an oblate ellipsoid (Cantor and Schimmel, 1980; Benecky *et al.*, 1990).

DISCUSSION

We have demonstrated that the λ repressor undergoes a concentration-dependent dissociation of tetramer to dimer in the concentration range of 20 to 0.2 μM. Previously, tetramer-dimer equilibrium of λ repressor has been studied by Chadwick *et al.* (1970) and Brack and Pirotta (1975), by sedimentation velocity and sedimentation equilibrium ultracentrifugation, respectively. Chadwick *et al.* (1970) showed that tetramerization occurs in the range of 10^{-7} to 10^{-5} M. Brack and Pirotta (1975) also showed that tetramerization occurs in this protein concentration range. None of the studies, however, gave an equilibrium constant. Our value of 2.3 ± 0.9 μM in 0.1 M phosphate buffer, pH 8.0, at 25 °C agrees with this range of dissociation. This indicates that modification of λ repressor by dansyl chloride has little effect on tetramer dissociation.

Previously, we have shown that operator binding to λ repressor causes a conformational change in the repressor which is transmitted to the C-terminal domain (Saha *et al.*, 1992). The significance of this conformational change is unclear, although we suspect that it may have some significance in protein-protein contact and binding co-operativity. The operator weakens the dimer-dimer interaction moderately, indicating that the conformational change may in some way be related to the effect of the operator on the dimer-dimer interaction. Similar weakening of dimer-dimer interaction has been reported in the lac repressor system (Royer *et al.*, 1990).

The co-operative interaction energy between two operator-bound repressor dimers are small (Koblans and Ackers, 1992), (Senear *et al.*, 1986). The co-operative interaction energies are the net result of several interactions including proximity effect, dimer-dimer interaction energy, DNA binding energy, etc. Slightly weaker protein-protein contact that occurs upon operator binding along with proximity effect may still be enough to overcome DNA and protein distortion energies and contribute to positive co-operative effects.

Rotational correlation times of the dimer and in particular the tetramer are substantially higher than perfectly spherical protein of same molecular weight. This indicates that the protein tumbles in solution as a whole and is unlikely to have two totally independently mobile domains. Previously, Banik *et al.* (1992) have shown on the basis of urea denaturation experiments that there are significant interactions present between C-terminal domain and hinge and N-terminal domain. Such interactions are likely to prevent the two domains to move totally independent of each other. The value of rotational correlation time obtained from Perrin plot is consistent with this fact. Recently, Hochschild and Ptashne (1988) have hypothesized that when the repressor is bound to operator, the C-terminal domain is loosely tethered to the N-terminal domain. Our fluorescence data indicate that such flexibility, if present, is unlikely to occur in the free repressor. In contrast, Weiss *et al.* (1987) have concluded from NMR experiments that the N-terminal domain is loosely tethered to the C-terminal domain and has mobility independent of the C-terminal domain. The NMR measurements, however, were done at much higher concentrations than our fluorescence anisotropy measurements and are likely to contain higher order oligomers. It is possible that higher oligomers

may have subtle structural and dynamical differences from the dimers and tetramers.

It is difficult to derive exact size and shape of dimers and tetramers, considering the inherent uncertainty. It is, however, clear that dimers are likely to be near spherical and the tetramer is likely to be quite elongated. The operators in the λ genome are separated by as much as 7 base pairs. An approximate distance calculation shows that the tetrameric repressor must be able to span 100 Å or more. Elongated tetramers deduced in this study would be consistent with the required ability of the repressor to span this distance.

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