Linkage between Operator Binding and Dimer to Octamer Self-Assembly of Bacteriophage λ *c*I Repressor[†]

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ABSTRACT: Cooperative binding of the bacteriophage λ *c*I repressor dimer to specific sites of the phage operators O_R and O_L controls the developmental state of the phage. Cooperativity has long been thought to be mediated by self-assembly of repressor dimers to form tetramers which can bind simultaneously to adjacent operators. More recently, we demonstrated that when free repressor dimers self-associate in solution, tetramer is an intermediate in a concerted assembly reaction leading to octamer as the predominant higher order species [Senear, D. F., et al. (1993) *Biochemistry 32*, 6179−6189]. Even as a minority component in the assembly reaction, tetramer can account for pairwise cooperativity. In a similar manner, were it able to bind all three operators simultaneously, octamer could account for three-way cooperativity. In fact, based solely on repressor self-assembly, the naive prediction is that the repressor−O_R interactions should be substantially more cooperative than they are. Evidently, there are unfavorable contributions to cooperativity from processes other than repressor self-assembly. Here, we focus on coupling between repressor self-association and operator binding as one possible unfavorable contribution to cooperativity. Sedimentation equilibrium analysis was used to compare the dimer−octamer association reactions of a repressor dimer−O_R1 complex and free repressor dimer. Fluorescence anisotropy was used to investigate O_R1 binding to free dimers and dimers assembled as higher order species. The results of these experiments indicate a significant and salt-dependent unfavorable contribution generated by such coupling. Since the oligonucleotides used in these experiments are the size of single operator sites, this coupling is mediated by the protein, not by the DNA. This mechanism does not account for an additional, salt-independent, unfavorable contribution which we presume is transmitted via the DNA. Thus, unfavorable contributions generated by structural transitions in both macromolecules serve to moderate the effect of self-association alone. We speculate that this is a general feature of cooperative protein−DNA interactions.

Cooperative binding of regulatory proteins to specific DNA sites is a ubiquitous feature of transcriptional regulation. The underlying molecular basis for cooperativity is believed almost universally to be provided by direct interactions between the DNA-bound proteins. Typically, the proteins involved in cooperative binding assemble when free in solution. The products of protein−protein assembly reactions are oligomeric complexes, and these can function as multidentate ligands for DNA binding.

The bacteriophage λ *c*I repressor protein is one model system for such interactions. Cooperative binding of *c*I repressor dimers to three adjacent sites at the λ right operator (O_R) constitutes the primary control of the switch between lysogenic and lytic phage cycles. Cooperativity is crucial to the regulation of this developmental switch. It confers stability to the lysogenic phase, while also providing rapid and essentially irreversible induction of lysis in response to damage to the host cell chromosome (Johnson et al., 1979; Shea & Ackers, 1985).

It is widely believed that cooperativity in the λ repressor binding to O_R involves self-association of repressor dimers to higher order oligomers, which then bind simultaneously to adjacent operators (Ptashne, 1992). This belief is founded on the demonstration of mass action assembly of free repressor dimers to higher order oligomers when the subunit concentration is in the micromolar range (Brack & Pirrotta, 1975; Pirrotta et al., 1970). The predominant high-order oligomer was long presumed to be tetramer (Ptashne, 1992), the species expected to mediate pairwise cooperativity between adjacent sites. However, more recently, this reaction was shown to be a concerted assembly of dimers to octamers (Senear et al., 1993). Whereas tetramer is an intermediate in the assembly process, it constitutes a relatively minor constituent under all conditions tested. This raises the question, what are the roles of tetramer and octamer in cooperativity?

Several macromolecular processes contribute to cooperative protein−DNA binding. The free energy change for protein−protein association contributes and, by itself, necessarily favorably. When oligomers interact simultaneously with multiple DNA sites, the unfavorable contribution from

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loss of translational and rotational entropy is paid only once, thus, increasing the probability of an oligomer binding to a second site when it is already bound to the first site. This contributes to what has been called the connection free energy (Jencks, 1981). But other interactions also can contribute to the connection free energy. For example, the oligomeric protein complex might not orient the different subunits' DNA binding surfaces in the optimal arrangement for interacting with adjacent operator sites. When this is the case, simultaneous interactions with multiple DNA sites require structural rearrangement of the protein, the DNA, or both. Conformational changes necessarily contribute unfavorably to cooperativity, even to the extent of precluding simultaneous interactions with multiple DNA sites. Finally, structural coupling between the different DNA binding units within the protein oligomer, as mediated by noncovalent interactions across subunit−subunit interfaces, can result in the different oligomeric forms having different intrinsic DNA binding properties. Such thermodynamic linkage between protein−protein assembly and DNA binding will contribute to cooperativity, either favorably or unfavorably.

Cooperative binding of separate proteins to adjacent DNA sites and assembly of the those proteins to oligomers, which then bind simultaneously, describe two paths around a thermodynamic cycle. Both result in the same end state, one in which the DNA sites are filled by an oligomeric protein complex. Consequently, the contribution from the protein assembly process to cooperativity can be assessed by comparing the free energy changes for opposite sides of the cycle: self-assembly and cooperativity. When applied to the cooperative *c*I repressor−O_R interactions, the appropriate comparison suggests that repressor tetramer can account for pairwise cooperativity, despite being a minor component of the assembly reaction (Senear et al., 1993). Similarly, octamer might account for a three-way cooperative interaction when all three operator sites are filled. In fact, the contribution from dimer−tetramer self-assembly would by itself account for much greater pairwise cooperativity than is observed (Senear et al., 1993). Similarly, the dimer−octamer self-assembly free energy would by itself account for much greater three-way cooperativity than is observed. Evidently there are compensating unfavorable contributions from other interactions.

In this report, we focus on thermodynamic linkage between repressor self-assembly and DNA binding as one source of unfavorable contributions. We have used sedimentation equilibrium to compare the dimer−octamer assembly reactions of free dimers to self-assembly reactions of dimers that are saturated with oligonucleotides containing the O_R1 sequence. We have also used the steady state anisotropy of 2-aminopurine-labeled O_R1 to investigate its binding to repressor dimers and higher order oligomers. By using single operator length oligonucleotides, the DNA bound to different repressor dimer units are separate molecules. Consequently, we anticipate that no structural rearrangement of either the protein or the DNA is required to accommodate simultaneous binding of multiple operators to these oligomers.

These experiments indicate that O_R1−liganded dimers still assemble in a concerted manner to form O_R1−liganded octamer, consistent with the results of a preliminary analysis (Laue et al., 1993). O_R1 binding neither dissociates the higher order oligomers to dimers nor does it substantially alter the stoichiometry to favor tetramers over octamers. However, O_R1 binding does significantly decrease the self-association of repressor dimers, an effect which is salt dependent. As in our earlier analysis of the self-association of free repressor, we were unable to evaluate precisely the proportion of tetramer that forms as an assembly intermediate. However, when the thermodynamic linkage between operator binding and self-assembly is projected on the dimer−tetramer assembly reaction, it predicts a substantial and unfavorable contribution to pairwise cooperativity. There is also a substantial contribution remaining, and this is independent of salt concentration. We presume that this reflects structural rearrangements required for simultaneous interaction of tetramers with adjacent operators on a continuous DNA helix.

MATERIALS AND METHODS

Repressor Preparation. Expression and purification of both Trp containing and 5-OHTrp containing wild-type λ *c*I repressor protein has been described previously (Ross et al., 1992; Senear et al., 1993). Neither repressor preparation contained a contaminating polypeptide that could be detected by Coomassie-stained SDS−polyacrylamide gels. Repressor concentrations were estimated from the 280 nm extinction coefficients, calculated from the average 280 nm extinction coefficients for tryptophan (5500 M^{-1} cm^{-1}) and tyrosine (1200 M^{-1} cm^{-1}) residues in a protein (Wetlaufer, 1962) and the 280 nm extinction coefficient of 5-OHTrp incorporated in a polypeptide (4500 M^{-1} cm^{-1}; Ross et al., 1992) assuming additivity of absorbances. Extinction coefficients at 230 and 310 nm were calculated from XL-A centrifuge absorption spectra (Laue et al., 1993) as described previously (Senear et al., 1993).

Operator DNA. The DNA fragments used in the sedimentation equilibrium experiments contained either 21 base pairs (bps) (O_R1−21) or 19 bps (O_R1−19) of the bacteriophage λ sequence centered on the 17 bp sequence of operator site, O_R1. The sequence of O_R1−21 is

$^{5'}$-TT**TACCTCTGGCGGTGATA**AT-$^{3'}$

$^{3'}$-AA**ATGGAGACCGCCACTAT**TA-$^{5'}$

Complimentary pairs of 5′-phosphorylated single-stranded deoxyribonucleotides, supplied by Oligos Etc, Inc., were annealed, and double-stranded operator was purified by chromatography on MonoQ as described previously (Laue et al., 1993). Purified operator DNA was homogeneous as assessed by ethidium bromide staining after polyacrylamide gel electrophoresis (15%, 29:1 acrylamide:bis(acrylamide) gels in 0.5× TBE buffer) and by sedimentation equilibrium analysis (see below).

O_R1 DNA used in the fluorescence experiments had the sequence of O_R1−21 shown above, except that the first T/A base pair was changed to A/T and 2-aminopurine (2-AP) was substituted for the 5′-A on both strands. The 2-AP-containing deoxyribonucleotides were synthesized as described (Fujimoto et al., 1996) and purified by reverse phase HPLC both before and after detritylation. These were annealed to double-stranded DNA (2-AP-O_R1−21) and purified as described above. 2-AP-O_R1−21 eluted from a MonoQ column (1 mL Pharmacia/LKB HR 5/5) as single peak at 0.53 M KCl. Small shoulders on the leading edge,

accounting for only several percent of the total material, were removed by the chromatography.

Sedimentation Equilibrium Experiments. High-speed sedimentation equilibrium experiments were conducted in a Beckman XL-A analytical ultracentrifuge using an absorbance optical system essentially as described (Senear et al., 1993). Repressor was prepared for centrifugation by exhaustive dialysis against 10 mM tris, pH 8.0, 2.5 mM $MgCl_2$, 1.0 mM $CaCl_2$, 0.1 mM dithiothreitol (assay buffer), and either 50 mM (low salt) or 200 mM (high salt) KCl. Stock DNA solutions were prepared by multiple rounds of ethanol precipitation and dissolution in assay buffer. Repressor concentrations loaded were 1.5, 0.5, and 0.15 mg/mL. O_R1 was loaded at a mole ratio of either 1.25 or 1.5 (as indicated) per mol repressor in dimer units. These solutions were centrifuged to equilibrium at multiple speeds, typically 10K, 14K and 20K rpm, at 23 °C.

Data were collected at intervals after estimated time to equilibrium and tested for equilibrium by subtracting successive scans (Yphantis, 1964). Channels containing only repressor protein were scanned at 280 and 230 nm; channels containing 5-OHTrp repressor were also scanned at 310 nm. Channels containing both repressor and O_R1 DNA were scanned only at 310 nm. Data within the optical window and linear absorbance range (Senear et al., 1993) were selected using the program REEDIT (kindly provided by David Yphantis). These were analyzed to estimate equilibrium association constants using the program NONLIN (Johnson et al., 1981). Multiple channels of sedimentation equilibrium data obtained at different wavelengths, loading concentrations, radial positions, and angular velocities were fit by global nonlinear least-squares to an assembly scheme that accounts for repressor dimers that assemble to form tetramers and octamers, according to

$$C = \Delta C_j + \exp(\ln C_o + \sigma(r^2/2 - r_o^2/2)) + \exp(\ln K_4 + 2(\ln C_o + \sigma(r^2/2 - r_o^2/2))) + \exp(\ln K_8 + 4(\ln C_o + \sigma(r^2/2 - r_o^2/2))) \quad (1)$$

C, in eq 1, is the repressor concentration (in absorbance or fringe displacement units) observed at radial position r, ΔC_j is a baseline offset for channel j, and C_o is the repressor dimer concentration at the meniscus (r_o). The reduced molecular weight, σ, is given by $\sigma = M_r(1 - \bar{v}\rho)\omega^2/RT$ (Yphantis & Waugh, 1956) where $\bar{v}$ is the partial specific volume, ρ is the buffer density, ω is the radial velocity, and R and T are the gas constant and absolute temperature, respectively. K_4 and K_8 are equilibrium association constants for the assembly of dimers to tetramers and of dimers to octamers, respectively. When chemical nonideality was considered, negative second virial coefficients were obtained consistently. Therefore, the self-assembly reactions were treated as ideal.

Conversion between reduced and nonreduced molecular weights used calculated buffer densities, $\rho = 1.04$ and 1.01 g/mL for 200 and 50 mM KCl (Laue et al., 1992) and partial specific volumes, $\bar{v} = 0.736$ mL/g for the repressor, calculated from the amino acid composition (Cohn & Edsall, 1943) and $\bar{v} = 0.55$ mL/g for the DNA (Durchschlag, 1986). The applicability of the latter value to small operator oligonucleotides under our solution conditions was assessed by conducting sedimentation equilibrium experiments on O_R1-19 and O_R1-21 alone under low-salt conditions. The results were consistent with those of homogeneous, noninteracting particles. When the data were analyzed using $\rho = 1.01$ and $\bar{v} = 0.55$ mL/g, apparent molecular weights 12 120 $\pm$ 1310 for O_R1-19 and 15 150 $\pm$ 1380 for O_R1-21 were obtained. The small average difference between these and the analytical molecular weights 11 740 and 12 980, are in the direction to underestimate the effect of operator binding on repressor octamer stability.

Fluorescence Experiments. The steady-state fluorescence anisotropy of constant concentrations of 2-AP-O_R1-21 was measured as a function of titrating repressor concentration using an SLM 4800 fluorometer modified for single photon counting. These binding titrations were conducted in assay buffer at 20 °C and either 75 mM or 200 mM KCl, as indicated. Light scattering due to a small fraction of aggregated material precluded making measurements at 50 mM KCl. The anisotropy $\langle r \rangle$ is expressed as

$$\langle r \rangle = \frac{I_v - I_h}{I_v + 2I_h} \quad (2)$$

where I_v and I_h are the intensities detected through vertical and horizontal polarizers when vertically polarized light is used to excite the sample. Excitation with horizontally polarized light was used to correct instrument bias in detection of the emission (Badea & Brand, 1979). The fluorescence emission was monitored at 380 nm with a band-pass of 16 nm using a monochromater. Excitation was at 315 nm with a band-pass of 8 nm using a monochromater. Fluorescence lifetimes were measured by time-correlated single-photon counting as described (Hasselbacher et al., 1991), using 315 nm excitation and 380 nm detection wavelengths.

The individual binding titrations conducted at each O_R1 concentration were analyzed separately to obtain apparent binding constants for operator binding to repressor dimers. The conservation polynomials for O_R1 and repressor were solved at each iteration in the nonlinear least-squares procedure using Newton's method as described previously (Senear & Ackers, 1990). The conservation equations accounted for repressor assembled as monomer and as dimer only, using reported values of the dimer k_d (Koblan & Ackers, 1991b). Higher order species are assumed implicitly by this analysis to be composed of dimeric units whose intrinsic operator binding affinity is the same as dimer. Subsequent global analyses accounted for repressor assembled as tetramer and as octamer in the conservation polynomials, using equilibrium constants for the assembly reactions reported previously (Senear et al., 1993). This generates conservation polynomials that are eighth-order in repressor concentration and fourth-order in operator concentration. These were solved at each iteration of the least-squares procedure using a Newton procedure modified with a line search (Press et al., 1992). Operator binding parameters pertaining to tetramer and octamer species are described in the text.

RESULTS

Self-Association of Free and Operator-bound Repressor. Our previous investigation of the higher order self-assembly of free repressor demonstrated a concerted assembly reaction

Table 1: Assembly Free Energy Changes for Free and O_R1−Bound Repressor Assembly at 200 mM KCl[a]

repressor	λ	ΔG_4[b]	ΔG_8[b]	$\Delta\Delta G_8$[c]	*s* (ODU)[d]
wild-type	280, 230		-23.1 ± 0.2		0.0053
	280, 230	-6.7 ± 0.2	-23.3 ± 0.1		0.0051
5-OHTrp	280, 230		-23.1 ± 0.2		0.0059
	280, 230, 310		-23.1 ± 0.2		0.0061
	280, 230, 310	-7.0 ± 0.1	-22.6 ± 0.1		0.0052
5-OHTrp/1.5 × O_R1	310		-21.9 ± 0.3	1.3 ± 0.4	0.0033
	310	-7.1 ± 0.1	-22.7 ± 0.3		0.0032

[a] Repressor protein was centrifuged to equilibrium at 10 000 and 20 000 rpm. Protein concentrations were determined as a function of radial distance by measuring the protein absorption at wave lengths of 230, 280, and 310 nm. Data obtained at different wavelengths were combined as indicated and analyzed according to dimer−octamer and dimer−tetramer−octamer assembly schemes using eq 1. [b] Free energy changes in kilocalories per mole relative to a 1 M standard state for assembly of dimers to tetramers (ΔG_4) and to octamers (ΔG_8). Values shown as ± are 65% confidence limits. [c] Difference in free energy changes for assembly of 5-OHTrp repressor dimers to octamers (ΔG_8) in the presence versus in the absence of a 1.5-fold mol excess of O_R1-21. [d] Square root of the variance of the fits in absorbance units.

of dimers to form octamers as the predominant assembly product in the micromolar concentration range (Senear et al., 1993). Dodecamers or other high molecular weight species that must predominate in the millimolar concentration range are negligible below 10^{-4} M. More significantly, the population of tetramers that form as an intermediate in the assembly reaction comprise no more than 10−20% of the total.[1]

Results of a preliminary analysis of the assembly properties of a repressor dimer, operator complex (R_2-O_R1) formed in the presence of a mole excess of O_R1 DNA, were also consistent with those of concerted, dimer−octamer assembly scheme (Laue et al., 1993). This indicated that binding to O_R1 does not result in octamers being simply dissolved to a mixture of dimers and tetramers. These results raise questions as to the potential roles of the higher order assemblies, both tetramer and octamer, in cooperative assembly of repressor−O_R complexes.

To address these questions, parallel sedimentation equilibrium analyses were conducted for repressor in the presence and absence of saturating O_R1. The repressor−O_R1 complex used 5-OHTrp repressor and O_R1-21 at 200 mM KCl. Data were collected at 310 nm, taking advantage of the red-shifted absorbance spectrum of 5-OHTrp to minimize interference from DNA absorbance (Laue et al., 1993). At this wavelength, the extinction coefficients of 5-OHTrp repressor dimer and O_R1-21 are approximately 17 400 and 1100 $M^{-1}cm^{-1}$, respectively, determined by absorbance ratios as described (Senear et al., 1993). Parallel experiments conducted on free repressor compared both wild-type and 5-OHTrp repressors, using separate cells in the same centrifuge rotor. To obtain data over the widest possible range of protein concentrations, these cells were scanned also at 280 and 230 nm.

Table 1 shows the results when these data were analyzed to obtain ΔG_8, the free energy change for dimer−octamer assembly, using an assembly model that accounts for octamer as the only self-association product of dimer (Senear et al., 1993). As shown previously, self-assembly of wild-type repressor and self-assembly of 5-OHTrp repressor are indistinguishable. For free 5-OHTrp repressor, ΔG_8 was not well resolved from the 310 nm data alone. This is because the extinction at 310 nm is relatively low, and, hence does not provide sufficient sensitivity to lower protein concentrations where the repressor is largely dimeric. However, the estimated value of ΔG_8 obtained from a multiwavelength analysis is the same whether or not the 310 nm data are considered, indicating that the data at all wavelengths are mutually consistent.

Data obtained using the 5-OHTrp, R_2-O_R1 complex were also analyzed according to the same dimer−octamer stoichiometry. For this analysis, the repressor was assumed to be saturated with operator at a stoichiometry of four O_R1 per octamer. The extinction and partial specific volume of the complex were both corrected for the contribution from the DNA based on this stoichiometry. In this case, analysis of the 310 nm data alone yielded a precise estimate of ΔG_8. The value obtained is more positive than for free repressor by 1.3 kcal/mol (Table 1), indicating a 10-fold decrease in stability for operator-bound repressor as compared to free repressor. This decreased stability shifts the assembly transition to higher repressor concentrations, and it is for that reason that the 310 nm data alone yield adequate resolution. Considering the thermodynamic cycle that relates repressor self-assembly and operator binding, a $\Delta\Delta G$ of 1.3 kcal/mol between the assembly of free and operator-bound dimers to form octamers corresponds to a binding affinity

[1] S. Roy and co-workers (Bandyopadhyay et al., 1996; Banik et al., 1993) have argued that the assembly stoichiometery is dimer to tetramer in the absence of divalent cations and have reported Van't Hoff enthalpy and entropy changes for dimer−tetramer assembly of both free (Banik et al., 1993) and O_R1−bound repressor (Bandyopadhyay et al., 1996) based on this interpretation. However, these authors also report light scattering data which contradict their interpretation (Bandyopadhyay et al., 1996). The intermediate plateau in the reduced scattering intensity data occurs at a value that is approximately $^1/_4$, not $^1/_2$, of the value obtained for octamer. Since the scattering intensity is proportional to the weight average molecular weight of the scattering particles (Cantor & Schimmel, 1980), a ratio equal to $^1/_4$ is consistent with dimer, not with tetramer. These data indicate relatively weak assembly of monomers to dimer, followed by concerted assembly of dimers to a species clearly larger than tetramer. To address the discrepancy between the two interpretations, we have conducted sedimentation equilibrium experiments on wild-type repressor using the buffer described by Roy. Under these conditions, we find a single assembly transition of dimers to a species with molecular weight about 200 000 (octamer) just as in the presence of divalent cations. The best fit to these data is dimer−tetramer−octamer with $\Delta G_4 = -7.2 \pm 0.1$ kcal/mol and $\Delta G_8 = -21.2 \pm 0.2$ kcal/mol. The midpoint of the transition is approximately 10−12 μM in repressor subunits. Consistent with both light scattering and large zone column chromatograpy data (Bandyopadhyay et al., 1996), we find that the primary effect of removal of divalent cations is to destabilize octamer, tetramer, and dimer. Although the proportion of subunits arranged as tetramer is greater in the absence than in the presence of divalent cations [maximum of approximately 40% versus 10−20%; (Senear et al., 1993)], the assembly of dimer to octamer is still concerted and there is no intermediate tetramer plateau. When the wrong assembly model is used and, as we show below, the O_R1 concentration is insufficient to saturate dimer, tetramer, or octamer, Van't Hoff analysis of apparent enthalpy and entropy changes is unreliable.

difference of 0.33 kcal/mol for each of the four O_R1 operators bound to the octamer. This is a significant, albeit modest, difference.

The centrifuge data were also analyzed using an assembly model that accounts for tetramer as an intermediate to the dimer−octamer assembly reaction. As described previously (Senear et al., 1993), there is extremely high numerical correlation between the parameters ΔG_4 and ΔG_8 that govern the assembly of free repressor dimers. In particular, if the values for either ΔG_4 or ΔG_8 obtained for free wild-type and 5-OHTrp repressor are compared to one another, they appear to differ significantly. Yet, the results from analysis according to the simpler dimer−octamer assembly model show that these two assembly transitions are indistinguishable. Thus, despite their apparent high precision, the separate values recovered from the numerical analysis for ΔG_4 or ΔG_8 are unreliable.

The values obtained for the 5-OHTrp R_2-O_R1 complex from the dimer−tetramer−octamer model suffer from similar correlation. Though an estimate for ΔG_4 is obtained, inclusion of this additional adjustable parameter in the fit results in no decrease in the variance. For other data sets, it was impossible to even obtain separate estimates of ΔG_4 and ΔG_8 (see below). Separate resolution might have been achievable had it been possible to employ either of the multiwavelength scanning techniques that have been described (Lewis et al., 1994; Schuck, 1994). Unfortunately, this is precluded by the high concentrations of both repressor and O_R1 necessary to drive complex formation and self-association, combined with the high UV absorbance extinction of DNA, as discussed previously (Laue et al., 1993). Nevertheless, the numerical correlation is a reflection of the small proportion of tetramers in the assembly reaction. Thus, it is possible to conclude from these results that the major assembly stoichiometry remains dimer−octamer; it has not become dimer−tetramer in the presence of DNA.

Effects of Salt Concentration and Operator Fragment Length. Electrostatic repulsion between separate ligand molecules is a possible explanation for the observation that O_R1−oligonucleotide binding destabilizes repressor oligomerization. Such a mechanism would be irrelevant to either tetramer or octamer binding to adjacent sites on the same DNA molecule. On the other hand, if tetramer or octamer can bind simultaneously to adjacent operators on the same DNA molecule, then the separate O_R1−oligonucleotides must be arranged end to end when bound to these repressor species in order to mimic a continuous helix. Consequently, the distance of closest approach between these molecules, hence the magnitude the electrostatic interaction, must be influenced substantially by the length of the bound oligonucleotides.

Parallel experiments were conducted using 19 and 21 bp O_R1 oligonucleotides (O_R1-19 and O_R1-21; see Materials and Methods) to assess the possibility of electrostatic end effects. To give the highest possible resolution of effects, experiments were conducted at 50 mM KCl (low salt) in addition to 200 mM KCL (high salt) as used above. The lower salt concentration has two advantages. First, pairwise cooperativity in dimer binding to adjacent operators sites is increased (Senear & Batey, 1991); the effect of operator binding on dimer self-assembly might be expected to increase commensurate with this. Second, electrostatic shielding is reduced, increasing sensitivity to potential electrostatic end effects.

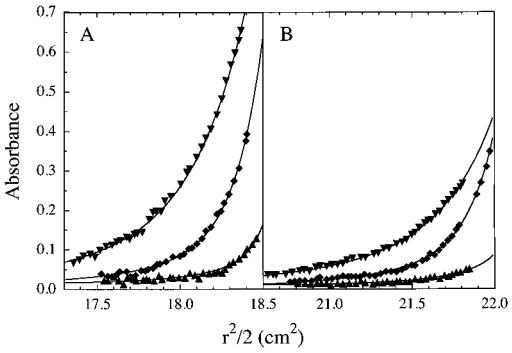

FIGURE 1: Sedimentation equilibrium data for the complex formed between 5-OHTrp repressor and O_R1-19, plotted as absorbance at 310 nm as a function of $r^2/2$. For clarity, only every third data point is shown. Experiments were conducted at 10 000 rpm (downward triangles), 14 000 rpm (diamonds), and at 20 000 rpm (triangles), 200 mM KCl and 23 °C. Panels A and B represent repressor loading concentrations of 1.5 and 0.5 mg/mL, each with a 1.25:1 mol ratio of O_R1-19 to repressor dimer. The solid curves drawn through the points represent the best fit to the association of dimers to octamers, with the dimer complex molecular weight fixed at the value of 64 150 determined from the amino acid and oligonucleotide sequences. Parameter values are in Table 2.

Table 2: Effect of Operator Binding on Free Energy Change for Repressor Dimer−Octamer Assembly[a]

[KCl] (mM)	operator	ΔG_8[b] rep (alone)	ΔG_8[b] rep-O_R1	$\Delta\Delta G_8$[c]
200	O_R1-21	-23.3 ± 0.2	-21.4 ± 0.7	1.8 ± 0.9
	O_R1-19	-23.1 ± 0.5	-21.4 ± 0.1	1.7 ± 0.5
50	O_R1-21	-23.3 ± 0.6	-19.6 ± 0.8	3.7 ± 1.0
	O_R1-19	-24.6 ± 0.7	-20.8 ± 1.0	3.8 ± 1.2

[a] Matched repressor solutions with and without a 1.25:1 mol ratio of O_R1 were centrifuged to equilibrium at 10 000, 14 000 and 20 000 rpm, using matched cells in the same centrifuge rotor. [b] Free energy changes in kilocalories per mole (± are 65% confidence limits) relative to a 1 M standard state for assembly of dimers to octamers (ΔG_8). [c] Difference in free energy changes for repressor dimers to octamers assembly in the presence versus in the absence of O_R1.

Figure 1 shows the radial mass distribution at sedimentation equilibrium for the complex formed between 5-OHTrp repressor and O_R1-19 at 200 mM KCl. For reasons described above, these data were analyzed according to a dimer−octamer assembly model. A parallel experiment was conducted on a matched solution of free 5-OHTrp repressor, in a different cell in the same centrifuge rotor. When compared using the analysis as outlined in Table 1, the data from this particular experiment yield $\Delta\Delta G_8 = 2.0 \pm 0.7$ kcal/mol.

Table 2 lists the results of comparisons between assembly of free 5-OHTrp repressor and the R_2-O_R1 complex for all four combinations of salt concentration and operator oligonucleotide length. The greater precision of the high-salt data reflects averaging of multiple experiments conducted for each operator oligonucleotide (O_R1-19 and O_R1-21). These results support three conclusions: first, the stability of higher order species of the R_2-O_R1 complex is significantly reduced compared to free repressor dimer; second, the effect on stability is significantly greater at low-salt conditions than at high-salt conditions; third, there is no effect of operator length at either salt condition. Therefore, the effect on the stability of the higher order repressor species is an allosteric effect mediated via interactions between the repressor dimers.

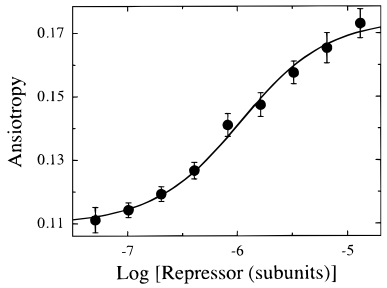

FIGURE 2: Steady-state fluorescence anisotropy of 2-AP-O_R1-21 at 75 mM KCl as it binds repressor. Error bars are standard deviations of means of multiple measurements. Curve drawn through the points shows the fit according to an $R \leftrightarrow R_2$ assembly model in which R_2 binds O_R1. This is equivalent to the assumption that repressor dimer units have the same O_R1 binding affinity, whether as free dimer or assembled as either tetramer or octamer (see text). This analysis yields a value $\Delta G_{app} = -8.5 \pm 0.2$ kcal/mol (Table 3). The standard state is 1 M.

It is not a result of electrostatic repulsion between bound operator oligonucleotides.

Repressor Binding to 2-Aminopurine-Labeled O_R1. Analysis of the sedimentation equilibrium data makes two assumptions: first, the repressor is saturated with O_R1; second, the binding stoichiometry is always 1 per unit of repressor dimer, whether that dimer is assembled as dimer, tetramer, or octamer. To evaluate these assumptions, binding of 2-AP-O_R1-21 was investigated directly at both low- and high-salt conditions using the steady state fluorescence anisotropy of 2-aminopurine. The low-salt condition employed a slightly higher KCl concentration then in the centrifuge experiments (75 mM KCl) to minimize interference from light scattering artifacts observed at 50 mM KCl.

Figure 2 shows increasing anisotropy as a fixed concentration of 2-AP-O_R1-21 (0.16 μM) is titrated with wild-type repressor at 200 mM KCl. This results from the increased rotational correlation time of the oligonucleotide when complexed with repressor. A significant quenching of the fluorescence yield was also noted (28%) resulting from a decrease in the fluorescence lifetime of the 2-aminopurine when 2-AP-O_R1-21 is bound to repressor. The decreased fluorescence lifetime also contributes to the increase in anisotropy of the steady state fluorescence of the probe.

To estimate the intrinsic affinity of repressor for 2-AP-O_R1-21, these data were analyzed according to a simple model that accounts for repressor as either dimer, which binds 2-AP-O_R1-21, or monomer, which does not (see Materials and Methods). At the higher repressor concentrations used in this experiment (up to 20 μM), tetramers and octamers are the predominant oligomeric repressor species. Consequently, this simple analysis assumes constant affinity of O_R1 for repressor dimer, whether assembled as dimer, tetramer, or octamer. In recognition of this simplification, we denote the free energy change obtained by this analysis, ΔG_{app}. We assumed similarly, that the fluorescence anisotropy of bound 2-AP-O_R1-21 was the same, whether bound to dimer or higher order species. This is as expected, given the short mean lifetime of the probe (3.7 ns; data not shown). We previously showed that even the slightly longer mean lifetime (4.5 ns) of the intrinsic tryptophan fluorescence of the repressor is short compared to the rotational correlation times of the larger repressor oligomers (Senear et al., 1993). Thus, while the steady state anisotropy does distinguish between bound and free 2-AP-O_R1-21, it does not distinguish among complexes with repressor species dimer and larger. The effect of fluorescence quenching was considered in accounting for the contribution of the bound 2-AP-O_R1-21 species to the observed anisotropy. As the O_R1 concentration used in this titration is not insignificant compared to the range of repressor concentrations that bind, it was also necessary to account for both O_R1−bound and free repressor in the analysis (see Materials and Methods).

Table 3: Repressor Binding to 2-AP-O_R1-21[a]

[O_R1] (μM)	ΔG(app)[b]	⟨*r*⟩ (free)[c]	⟨*r*⟩ (bound)[c]	*s*[d]
200 mM KCl				
0.16	-8.5 ± 0.18	0.1099 ± 0.0022	0.1747 ± 0.0034	0.0023
75 mM KCl				
0.16	-9.9 ± 0.13	0.1113 ± 0.0015	0.1721 ± 0.0013	0.0014
0.16	-9.1 ± 0.34	0.1092 ± 0.0053	0.1715 ± 0.0043	0.0044
0.40	-9.1 ± 0.26	0.1169 ± 0.0036	0.1906 ± 0.0056	0.0041
0.88	-9.1 ± 0.23	0.1120 ± 0.0023	0.1915 ± 0.0044	0.0025
1.00	-9.1 ± 0.25	0.1120 ± 0.0014	0.1846 ± 0.0042	0.0019
1.00	-9.8 ± 0.30	0.1188 ± 0.0016	0.1862 ± 0.0032	0.0019
1.68	-8.7 ± 0.24	0.1027 ± 0.0024	0.1774 ± 0.0033	0.0025
2.50	-8.5 ± 0.26	0.1127 ± 0.0025	0.1971 ± 0.0053	0.0030
5.00	-8.0 ± 0.42	0.1116 ± 0.0028	0.1960 ± 0.0142	0.0035

[a] Steady state anisotropy of 2-AP-O_R1-21 was monitored while titrating fixed concentrations of operator as shown with repressor. [b] Apparent free energy changes in kilocalories per mole for 2-AP-O_R1-21 binding to wild-type repressor, assuming the same intrinsic affinity for binding to all repressor dimer units, whether assembled as dimer, tetramer, or octamer . [c] Fitted titration endpoints (± are 65% confidence limits) corresponding to the steady state anisotropy of free and repressor-bound 2-AP-O_R1-21. [d] Square root of the variance of the fitted curves.

Results from this analysis of titrations conducted at both 75 and 200 mM KCl are reported in Table 3. Values obtained for $K_{dssn,app}$ are 8.1×10^{-8} M and 4.5×10^{-7} M, respectively. Considering the simplifications in the analysis, these may underestimate slightly the O_R1 binding affinity of dimers. The salt dependence is consistent in direction, though less in magnitude, with repressor binding to site O_R1 in the context of the full-length λ O_R (Koblan & Ackers, 1991a; Senear & Batey, 1991). Taking into account the effect of the observed salt dependence to predict $K_{dssn,app}$ at 50 mM KCl gives a value more than 2 orders of magnitude below the lowest concentration of repressor dimer used as a cell loading concentration in the centrifuge studies. Thus, under low-salt conditions, the repressor was always over 99% saturated with O_R1. At 200 mM KCl, a similar analysis indicates a minimum of 95% saturation.

A series of additional titrations was conducted at 75 mM KCl using different fixed 2-AP-O_R1-21 concentrations (Figure 3). These experiments were conducted for two reasons. First, as the O_R1 concentration is increased, hence also, the total repressor concentration needed to saturate the DNA, the equilibrium mixture of repressor species is increasingly populated by tetramers and octamers as opposed to dimers. Consequently, thermodynamic linkage between self-assembly of repressor dimers and O_R1 binding should result in a systematic decrease in the apparent O_R1 binding affinity as its concentration is increased over a series of titrations. Second, even with such linkage, it might be possible to attain a sufficient O_R1 concentration that repressor binds stoichiometrically. This is useful to determine the O_R1 binding stoichiometry of octameric repressor.

Separate analysis of each of these binding curves using the simple model described above indicates a systematic decrease in apparent binding affinity as the 2-AP-O_R1-21

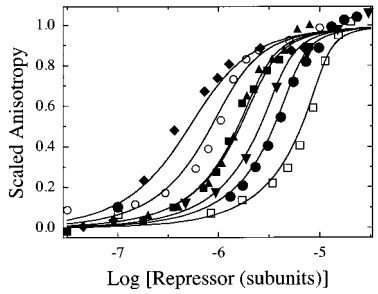

FIGURE 3: Steady-state fluorescence anisotropy of 2-AP-O_R1-21 at 75 mM KCl as it binds repressor. A series of titrations is represented, each conducted at a fixed concentration of 2-AP-O_R1-21. Concentrations of 2-AP-O_R1-21 are 0.16, 0.4, 0.88, 1.0, 1.7, 2.5, and 5.0 μM. Curves drawn through the points show the fit according to an $R \leftrightarrow R_2 \leftrightarrow R_8$ assembly model in which R_2 and R_8 bind O_R1 but with different affinities. In this model, O_R1 binding to R_8 is noncooperative (see text) but assembly of R_2 to R_8 decreases the affinity for O_R1. Similar curves are obtained using a model in which R_2 and R_8 have the same intrinsic affinity for O_R1, but O_R1 binding to R_8 is negatively cooperative. Parameter values are in Table 4.

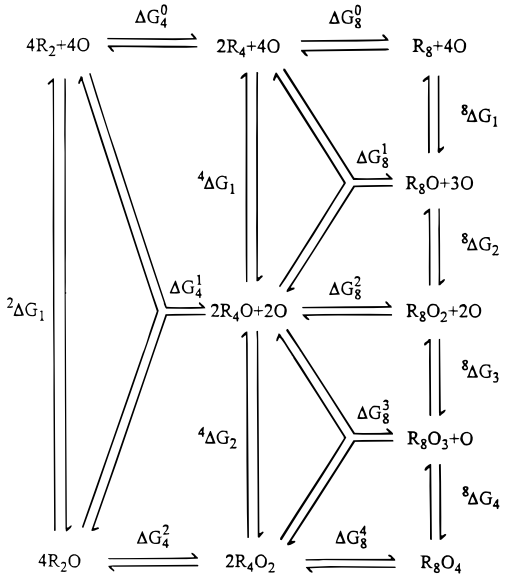

FIGURE 4: Linkage of O_R1 oligonucleotide and self-assembly of repressor dimers. O denotes O_R1-21 or O_R1-19. R_2, R_4, and R_8 denote repressor dimer, tetramer, and octamer. The free energy changes assembly of free and O_R1−liganded dimers to tetramer are denoted by ΔG_4^i, where the subscript refers to 0, 1, or 2 O_R1 oligonucleotides bound. Similarly the free energy changes for assembly of *dimers* to octamer are denoted by ΔG_8^i. The stepwise free energy changes for successive O_R1 oligonucleotide binding to dimers, tetramers, and octamers are denoted by $^2\Delta G_i$, $^4\Delta G_i$, and $^8\Delta G_i$, respectively. Reprinted with permission from Senear, D. F., et al. (1993) *Biochemistry 32*, 6179−6189. Copyright 1993 American Chemical Society.

concentration is raised above 1 μM (Table 3), consistent with the results of the sedimentation equilibrium studies. There isno indication of an asymptotic limit to the apparent affinity at the highest 2-AP-O_R1-21 concentration used (5 μM). This limit would provide an estimate of the affinity of 2-AP-O_R1-21 for binding to repressor octamers. Unfortunately, 5 μM is the highest O_R1 concentration for which the fluorescence anisotropy titration is technically feasible. The repressor concentrations needed to saturate at this operator concentration approach the limit where the assumption that octamer is the largest repressor oligomer is no longer accurate (Senear et al., 1993). However, it is apparent that the binding curve at 5 μM O_R1 is approaching the limit of a site titration. The saturation point in the this curve is very close to 10 μM repressor subunits, consistent with a stoichiometry of 1 O_R1 per dimer. At this concentration of free repressor, octamer is the predominant oligomeric form. From this we infer that 1 O_R1 per dimer unit is the binding stoichiometry for both octamer and dimer as suggested previously (Laue et al., 1993).

To obtain an estimate of the affinity of 2-AP-O_R1-21 for binding to repressor octamers, all of the anisotropy data were analyzed globally using models that account for the thermodynamic linkage. The complete linkage scheme that relates self-assembly of dimers to tetramers, and octamers, to operator binding by dimers, tetramers and octamers, is represented in Figure 4. This accounts for the possibilities that each oligomeric species might differ in its intrinsic binding affinity and that binding to tetramer and octamer might be cooperative (e.g., $^8\Delta G_1 \neq {}^8\Delta G_2 \neq {}^8\Delta G_3 \neq {}^8\Delta G_4$). These possibilities were considered separately using simplifying assumptions. In the first case, binding to octamer was assumed to be noncooperative (i.e., $^8\Delta G_1 = {}^8\Delta G_2 = {}^8\Delta G_3 = {}^8\Delta G_4$) to differ in intrinsic affinity from binding to dimer (i.e., $^8\Delta G_1 \neq {}^2\Delta G_1$). In the second case, these assumptions were reversed ($^8\Delta G_1 = {}^2\Delta G_1$), and cooperativity was assumed to be constant at each binding step (i.e., $^8\Delta G_{i+1} = {}_8\Delta G_i + {}^8\Delta G_{coop}$). Tetramer was not considered.

Parameter values obtained from both analyses are in Table 4. These two models yield indistinguishable binding curves which are shown plotted in Figure 3. Considering the linkage

Table 4: Global Analysis of Repressor Binding to 2-AP-O1−21[a]

$^2\Delta G_1$[b]	$^8\Delta G_1$[b]	$^8\Delta G_c$[b]	$\Delta\Delta G_8$[c]	s[d]
Noncooperative Binding to Octamer				
-9.8 ± 0.21	-9.2 (>-9.8)		2.4	0.0034
Cooperative Binding to Octamer				
-9.8 ± 0.21		0.9($>$0.1)	2.7	0.0034

[a] Data in Figure 3 analyzed using noncooperative and cooperative binding models described in the text. [b] Gibbs free energy changes (kcal/mol) with 65% confidence limits for intrinsic O_R1 binding to dimer ($^2\Delta G_1$) and octamer ($^8\Delta G_1$), and cooperative free energy change for binding of each O_R1 beyond the first to octamer. [c] Difference in total free energy change to saturate octamer versus four dimers with O_R1; equivalent to $\Delta\Delta G_8$ in Tables 1 and 2. [d] Square root of the variance of the fitted curves.

scheme, $\Delta\Delta G_8$ is calculated as the difference between the total free energy changes to saturate either octamer or four dimers with O_R1. The two models yield similar values of $\Delta\Delta G_8$. Considering the confidence limits for the binding parameters, these models do not clearly eliminate the possibility that assembly to octamer has only a negligible effect on binding. This imprecise resolution of effects is a feature of the models used rather than the data themselves since a systematic trend in the direction of weaker binding is evident in the apparent binding free energies in Table 3. The confidence limits also allow the possibility that octamer does not bind O_R1 at all. However, this is refuted by the sedimentation data where there is a clear rise in the molecular weight when operator is added, consistent only with O_R1 binding to octamer. The maximum likelihood values determined from the anisotropy data at 75 mM KCl are intermediate between values obtained from the centrifuge results at 200 mM KCL and at 50 mM KCL. Thus, the

consistency between the two experimental methods and over a range of KCl concentrations is remarkable.

DISCUSSION

Cooperative protein−DNA interaction is a widespread phenomenon that is of particular significance to transcriptional regulation. The functional reasons for cooperativity include providing high sensitivity to changes in active concentrations of regulatory proteins, recruiting of proteins that otherwise bind DNA either weakly or relatively nonspecifically, and stimulating the enzymatic activity of RNA polymerase. The driving force for cooperative assembly of protein-DNA complexes is thought to be provided by direct protein−protein association in most cases. But other interactions mediated by both the proteins and DNA are also involved. Despite the widespread significance of cooperativity to transcriptional regulation, the complete set of interactions and balance of forces they provide is not known for any regulatory system.

The λ phage repressor is a prototype for such interactions. The protein has separate N- and C-terminal domains for DNA-binding and self-assembly. A goal of our studies has been to describe the balance of forces governing the cooperative assembly of repressor−operator complexes. Our strategy has been to compare the energetics of self-assembly of repressor dimers when free in solution to the assembly energetics of dimers bound to single-operator and multiple-operator DNA. We described the assembly of free repressor dimers previously (Senear et al., 1993). Here, we have described the assembly of dimers when bound to single operators.

A surprising outcome of the earlier study is that repressor dimers assemble in a concerted manner to form octamers as the predominant higher order species. This finding raised the question whether both tetramer and octamer play roles in cooperativity, e.g., by mediating pairwise and three-way interactions, respectively. The high self-affinity of dimers to form higher order oligomers in solution raised a second question of why dimer binding to O_R and O_L is not substantially more cooperative than it is. To illustrate this point, consider the free energy changes for assembly of free dimers to tetramer [$\Delta G_4 = -7$ kcal/mol at 200 mM KCl (Senear et al., 1993)] and for pairwise cooperativity between dimers bound to adjacent operators [e.g., $\Delta G_{12} = -2.8$ kcal/mol (Senear & Batey, 1991)]. Assembly in solution has to pay the price of loss of translational and rotational (and probably conformational) entropy, whereas for bound dimers, this price has presumably already been paid upon binding to DNA. Yet, contrary to what would be expected based on this reasoning, tetramer formation between free dimers is more probable than between dimers already bound to adjacent operators.

By exactly the same difference, intrinsic (i.e., noncooperative) binding of separate dimers to adjacent operators is more probable than binding of tetramer. The entropic advantage of binding a multidentate ligand (tetramer) versus separate binding of its parts (dimers) is sometimes used to calculate a "local concentration" of the multidentate ligand when one of its parts is already bound (Creighton, 1993). From this analysis, the local concentration of tetramer bound to an operator site as seen by the adjacent operator is approximately 1 mM. This effective concentration, calculated from standard free energy changes, refers to a 1 M standard state concentration of complex. An effective concentration less than 1 M reflects the fact that the connection free energy (Jencks, 1981) must be a positive term; the effect of anchoring the tetramer by binding to one operator on the interaction between tetramer and the second site is unfavorable.

As outlined in the introductory portion of this paper, two general types of interactions can explain this behavior. The first is allostery mediated by noncovalent interaction across the dimer−dimer interface of the higher order oligomeric species (tetramer and octamer) when free in solution. Formation of tetramers may increase or decrease the intrinsic DNA affinity of each subunit relative to that of free dimer. Second, a higher order protein oligomer can interact simultaneously with adjacent operators only when the spacing and orientation of the adjacent operators on the same DNA molecule matches that of the dimers in the oligomer. If this is not the case for free tetramers and O_R1 DNA in solution, then one or the other or both must change conformation to form a complex at both sites. The contribution from this process to cooperativity is necessarily unfavorable.

The studies we report here assess contributions from the first of these processes, dimer−dimer interaction. Since the operator oligonucleotides bound to separate dimer units within repressor tetramers and octamers are separate molecules, the requirement for proper orientation is obviated. Our results indicate a substantial contribution from dimer−dimer interactions, accounting for as much as 3.8 kcal/mol for octamer at 50 mM KCl. It would be of interest to know what portion of this energetic penalty is paid at the level of tetramer formation. A complication is that our results do not resolve the free energy change for tetramer formation (ΔG_4). However, the results do indicate that tetramer remains a minor component of the assembly transition of O_R1-bound dimers, just as for free dimer. We infer from this that allosteric interactions in the tetramer must also decrease its intrinsic operator binding affinity, similar to octamer. If we assume that the relative proportions of tetramer and octamers species is the same for free versus operator-bound repressor, then $\Delta\Delta G_4$ should equal one half of $\Delta\Delta G_8$, or about +0.9, 1.3, and 1.9 kcal/mol at 200, 75, and 50 mM KCl, respectively.

According to the classic allosteric models, reciprocal constraints imposed on dimer units when assembled as tetramer and/or octamer would affect intrinsic DNA-binding affinity. These constraints would be relieved in a stepwise fashion as operator-binding sites are filled, leading to cooperative oligonucleotide binding. The analyses reported in Table 4 do not resolve whether O_R1 oligonucleotide binding to octamer is cooperative (positive or negative). The data are consistent with either different intrinsic DNA-binding affinities of dimer and octamer or negative cooperativity in O_R1 oligonucleotide binding to octamer. The two models yield similar estimates of $\Delta\Delta G_8$. Albeit with broad confidence limits, these best estimates at 75 mM KCl are exactly intermediate between the estimates at 50 and 200 mM KCl obtained using an entirely different experimental approach.

It is clear that if octamer binds simultaneously to three operators, it does so with a free energy change that is substantially less then the sum of intrinsic free energy changes for dimer binding separately to the three sites. If

this were not the case, dimer and tetramer could not compete with octamer for binding. O_R would be a simple two-state switch, empty or full, and there would be no differential regulation of the divergent promoters, P_R and P_{RM} (Johnson et al., 1979; Shea & Ackers, 1985). At all conditions examined here, the $\Delta\Delta G_8$ we obtain is far less than needed to explain why octamer doesn't saturate O_R in a single step.

We presume that there must be a substantial unfavorable contribution resulting from conformational changes necessary to facilitate simultaneous interaction of octamer with all three operators. For octamer, the magnitude of this effect is much greater than the allosteric contribution. Evidently, the dimer units are not arranged as necessary for simultaneous interaction with three operators arranged adjacent to one another on the same DNA molecule. If this is the case, then octamer is unlikely to be involved directly in cooperative binding. As suggested previously (Senear et al., 1993), one possible role of octamer is to buffer the concentrations of the relevant species (dimer and tetramer) at levels consistent with those needed for specific binding and cooperativity, but at levels low enough to prevent significant non-specific DNA binding (where pairwise cooperativity between adjacent dimers also might be expected).

Similarly, the contribution to tetramer formation, while significant, does not by itself account for the observed pairwise cooperativity. Again, a substantial contribution must come from conformational changes. There is an interesting correlation between $\Delta\Delta G_4$ and the pairwise cooperativity (ΔG_{ij}) when compared as a function of KCl concentration. The former increases in magnitude as the KCl concentration decreases. Given relatively constant free energy change for assembly of free dimers to tetramer [$\Delta G_4 \approx -6.7$ to -7 kcal/mol; (Senear et al., 1993)], this dependence is in the correct direction to explain why cooperativity decreases in magnitude with decreasing KCl concentration (Senear & Batey, 1991). After accounting for the allosteric contribution, the remainder of the difference between ΔG_4 and ΔG_{ij}, amounting to 2.7−3.1 kcal/mol, is essentially independent of KCl concentration. This must be an effect resulting from distortion of either or both interacting macromolecules.

We conclude that cooperativity in *c*I repressor−O_R interactions represents a balance between the favorable contribution from self-assembly of dimers in solution, and unfavorable contributions from both distortions of either or both the free DNA and free repressor oligomer structures and a salt-dependent effect of repressor self-assembly on intrinsic operator binding affinity. An interesting property that emerges from this accounting is that the connection free energy is unfavorable. Multidentate binding is often used to drive DNA topological transitions such as loop formation. In these cases, the unfavorable free energy associated with the DNA structural change is substantial; this can result in unfavorable connection free energies even for very stable oligomeric proteins [*cf.* Brenowitz et al. (1991)]. But unfavorable connection free energy has also been noted for the intramolecular coupling between separate DNA-binding domains in the same protein which, like the λ dimers, bind to adjacent motifs. For example, results obtained for the Oct-1 POU domain, which is comprised of separate POU-specific and POU homeodomains, yield an effective concentration of several millimolar (Klemm & Pabo, 1996). Considering that the standard state is 1 M, this is a positive connection free energy. A more dramatic example is provided by the PAX transcription factors (Underhill et al., 1995) in which individual PAX paired- and homeodomain DNA recognition motifs have been observed to bind with similar affinity as the complete DNA target site. In this case, the linking peptide completely negates the favorable binding contributions from the second domain.

Coupling between favorable protein−DNA interactions and unfavorable conformational transitions, such as protein folding, is common to transcriptional regulatory proteins. This has been proposed as a mechanism to provide simultaneously both the high specificity required of regulatory protein−DNA interactions and moderate binding affinities, suitable to biological regulation (Sauer, 1990). Following similar reasoning, it typically requires only a few kilocalories per mole in cooperative free energy change to approach the functional limit of infinite cooperativity. Yet, as a free energy change governing assembly of protein subunits in solution, such weak interaction is probably insufficient to provide the necessary specificity. Perhaps coupling of intrinsically high-affinity intermolecular interactions to unfavorable conformational transitions is a more widespread phenomenon than appreciated previously.

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