Self-Assembly of Bacteriophage λ *c*I Repressor: Effects of Single-Site Mutations on the Monomer–Dimer Equilibrium†

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ABSTRACT: Dimerization of λ *c*I repressor monomers is required for high-affinity binding to bacteriophage λ operator DNA and is known to involve protein–protein contacts between C-terminal domains of the repressor monomers. In order to address the importance of the C-terminal domain in mediating the oligomeric properties of dimerization and cooperative binding to operator DNA, eight single-site mutant repressors were screened for possible deficiencies in cooperative interactions; all but one of the amino acid substitutions are located within the C-terminal domain. As a prelude to binding studies and the complete characterization of cooperativity mutants of λ *c*I repressor (Burz, D. S., & Ackers, G. K. (1994) *Biochemistry 33,* 8406–8416), the thermodynamics of self-assembly of seven of these mutants was examined from 10^{-11} to 10^{-5} M total repressor using analytical gel chromatography. Results show that the structural perturbation accompanying single amino acid replacement does not significantly affect the monomer–dimer equilibrium with the exception of that accompanying replacements of serine 228; mutations at that site weaken, by 2–4 kcal/mol, the protein–protein interactions responsible for self-association. An additional mutant repressor, Pro158→Thr, was also examined and found to associate reversibly from monomers to a species with stoichiometry greater than 2. All mutations increase the apparent Stokes radius of the monomeric form by 2–4.5 Å and that of dimers by 1 or 3 Å.

The binding of many transcriptional regulatory proteins to specific DNA sequences is energetically coupled to the assembly of high-affinity species. In bacteriophage λ, the regulation of gene expression is manifested through cooperative binding of *c*I repressor *dimers* to the six sites on the right and left operators (O_R and O_L) of the phage genome [Johnson et al., 1979, 1981; for a review, see Ptashne (1992)]. Interactions involving *c*I repressor and the right operator are shown in Figure 1. Monomers are in dynamic equilibrium with dimers, which are the binding species (Chadwick et al., 1973; Sauer, 1979; Beckett et al., 1991). Dimers bind to individual operator sites with intrinsic free energy, while additional binding free energy is provided by cooperative interactions between adjacently bound dimers. Characterization of the interaction of repressor with the right operator is thus determined by seven energetic terms: three intrinsic free energies of interaction of repressor dimers with individual operator sites, three cooperative free energy terms, and the free energy of repressor dimerization. Repressor monomers dimerize with −11 kcal/mol of free energy, while the intrinsic affinity of *c*I repressor dimers for operator DNA ranges from −10 to −13 kcal/mol. Dimerization and operator binding are thus energetically coupled, and a correct interpretation of binding data must consider the free energy contributions of linked processes in the final analysis.

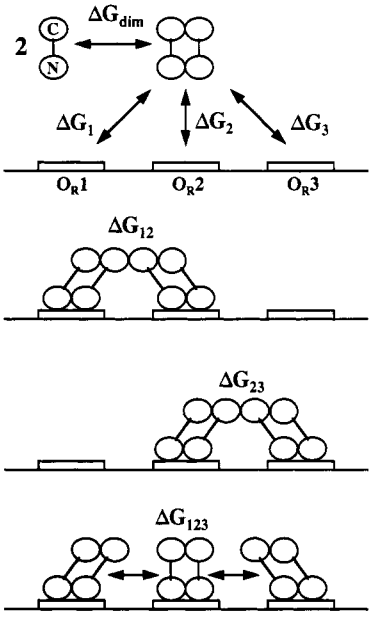

FIGURE 1: Interactions involving *c*I repressor and the right operator (O_R). O_R contains three 17 base pair operator sites, O_R1, O_R2, and O_R3, spaced at 7- and 6-bp intervals, respectively. The dumbbell depiction of *c*I repressor reflects its domain structure: The N-terminal domain (residues 1–102) mediates protein–DNA contacts, while self-association and cooperative DNA binding of *c*I repressor is known to involve protein–protein contacts between C-terminal domains (residues 134–236) of the repressor monomers (Pabo et al., 1979).

The exact role of cooperative repressor interactions in the biological function of bacteriophage λ has not been accessible to direct experimental investigation because mutants defective in cooperative binding but competent in dimerization and binding to individual operator sites (*i.e.*, cooperativity mutants) have not, until recently, been available. Genetic screening of *c*I repressors selectively mutagenized in the C-terminal domain led to the isolation of single amino acid substituted candidates with possible defects in cooperative behavior (Beckett et al.,

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1993). Genetic screens designed to select for spontaneous λ *c*I cooperativity mutants, based on the loss of ability to control expression of an operon due to deficiencies in pairwise cooperative recognition of tandem operator sites, yielded additional repressor proteins whose mutations were confined exclusively to the C-terminal domain (Benson et al., 1994). Both approaches verified that the mutated proteins were capable of binding to a single operator site by regulating the expression of downstream coding sequences and by the ability to confer immunity to superinfection against a series of virulent phages.

The initial screening of mutant repressors reported in Beckett et al. (1993) used nitrocellulose filter binding to compare the affinity of the proteins isolated for single- and two-site operator DNA with that of wild type. A mutant repressor deficient only in its ability to self-associate to dimers will exhibit weaker apparent affinity for a multiple-site operator, relative to wild type, when affinity is measured as a function of total monomer. In order to distinguish between mutations which specifically affect monomer–monomer interactions and/or cooperative interactions between dimers adjacently bound to operator DNA, the monomer–dimer equilibrium constants were determined in this study for six mutant repressors: Glu102→Lys (EK102), Gly147→Asp (GD147), Lys192→Asn (KN192), Tyr210→His (YH210), Ser228→Arg (SR228), and Ser228→Asn (SN228). In the following paper, we consider the energetic contribution of dimerization to individual site binding isotherms obtained for the interaction of single-site mutants of *c*I repressors to O_R. A preliminary account of this work has been presented elsewhere (Burz et al., 1993).

EXPERIMENTAL PROCEDURES

Chemicals and Biochemical Materials. Ampicillin, bovine serum albumin (BSA), isopropyl β-D-thiogalactoside (IPTG), lysozyme, Nonidet P-40, phenylmethanesulfonyl fluoride, polyethylenimine, and Sephadexes were from Sigma Chemical Co. Electrophoresis grade acrylamide, ammonium persulfate, bis(acrylamide), and TEMED were obtained from Bio-Rad. Acrylamide and bis(acrylamide) were deionized using Bio-Rad AG501-X8 mixed-bed resin prior to use.

Tran-^{35}S label (1100 Ci/mmol) was from ICN Biomedicals, Inc. Deoxyribonuclease I (bovine pancreas, code D) was from Worthington. Affi-Gel Blue affinity resin was from Bio-Rad. Extracti-Gel D resin was from Pierce or P.J. Cobert. Hydroxyapatite-Ultrogel (HAP-Ultrogel) was from IBF Biotechnics. Centriflo membrane cones (CF25) were from Amicon. All other chemicals were reagent or analytical grade.

Molecular size standards α-chymotrypsinogen A (bovine pancreas, type II), serum albumin (bovine, fraction V), cytochrome *c* (equine heart, type VI), myoglobin (equine skeletal muscle, type I), and ovalbumin (chicken, grade VI) used in calibrating the column and in determining Stokes radii were from Sigma.

Protein Purification. Repressors were overexpressed from the *tac* promoter on plasmid pEA3000 (Amman et al., 1983) for wild type or on pFG600 (Gimble & Sauer, 1989) for the mutants (Beckett et al., 1993) and purified from *Escherichia coli* strain X90 as described (Johnson et al., 1980). The purification properties of the mutant repressors were similar to those of wild type, while the yields varied over a considerable range. All preparations were deemed >95% pure by Coomassie Brilliant Blue staining of samples electrophoresed on 8% SDS–polyacrylamide gels (Laemmli, 1970). Total monomer concentration of unlabeled repressor was measured by UV absorbance at 280 nm, assuming an extinction coefficient of 1.18 mL mg^{-1} cm^{-1} (Sauer & Anderegg, 1978).

Radiolabeled repressors used in the determination of monomer–dimer equilibrium constants were prepared according to the method described in Beckett et al. (1991) from *E. coli* strain RB791 [W3110*lac*I^{Q}L8] (Brent & Ptashne, 1981). The purification properties of the ^{35}S-labeled proteins differed slightly from those of wild type. Most of the mutant repressors eluted from Affigel Blue resin at a KCl concentration of ~0.4 M; SR228 eluted from this resin at 0.8–1.0 M. Similarly, most of the proteins eluted from hydroxyapatite (HAP-Ultrogel) at 0.4 M potassium phosphate; however, wild type eluted at a slightly higher phosphate concentration, while YH210 and SN228 eluted at ~0.2 M. The yields of EK102, GD147, KN192, and SN228 were comparable to that obtained for wild type, whereas the yields of PT158, YH210, and SR228 were about 10% that of wild type. Specific activities of radiolabeled repressors were ~10^{17} cpm/mol except for those of PT158 and YH210, which were ~5 × 10^{15} cpm/mol. Labeled proteins were greater than 95% pure as estimated from autoradiography and silver staining of Laemmli gels of electrophoresed samples. Concentration was determined using a micro Bradford assay [Hammond & Kruger, 1988; *cf.* Bradford (1978)]. All repressor preparations were stored at −70 °C.

Analytical Gel Chromatography. Determination of the monomer–dimer equilibrium constant for each mutant repressor was accomplished using analytical gel chromatography [Ackers 1970, 1975; Valdes & Ackers, 1979; Beckett et al., 1991; *cf.* Koblan and Ackers (1991)]. Large-zone (30 mL) experiments were carried out on a 0.9 × 40 cm Sephadex G-100 column as described in Beckett et al. (1991). Experiments were performed over a range of concentrations from 10^{-12} to 10^{-5} M in repressor monomer in buffer containing 10 mM Bis–Tris (pH 7.00 at 20 °C), 200 mM KCl, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, and 100 μg/mL BSA. High-concentration zones were prepared by weight dilution of unlabeled repressor into buffer followed by the addition of labeled repressor at a very low concentration (<10^{-9} M); low-concentration zones were prepared by volume dilution of stock radiolabeled repressor into buffer. Flow was maintained at 0.25–0.29 mL/min using an LKB 10200 Perpex peristaltic pump fitted with silicon rubber tubing (LKB). Flow rates were determined by the weight average of every twentieth fraction of column eluant and found to vary ±0.05 mL/min over the course of a single experiment. One-minute fractions of eluant were collected, and elution profiles were determined by liquid scintillation counting of the fractions in 4 mL of Aquasol-2 (National Diagnostics or New England Nuclear).

Analysis of Chromatographic Data. Elution volumes (V_e) of large zones of *c*I repressor were determined as the equivalent sharp boundaries (centroids) of the leading edges of the zone by numerical integration [Figure 2; see also Valdes and Ackers (1979)]. Weight average partition coefficients (σ_w) were calculated relative to totally excluded (blue dextran) and included (tryptophan) standards by

$$\sigma_w = (V_e - V_o)/V_i \quad (1)$$

where V_o and V_i are the void and internal volumes of the column, typically 9.5–10.5 and 31.5–34 mL, respectively. The resulting data, σ_w vs [*c*I monomer], were fit to a monomer–dimer stoichiometric model by a nonlinear least-squares technique (Johnson et al., 1976), using the following equations:

$$\sigma_w = f_1\sigma_m + (1 - f_1)\sigma_d \quad (2)$$

$$f_1 = (-K_{dim} + (K_{dim}^2 + 8K_{dim}C_T)^{1/2})/4C_T \quad (3)$$

where σ_m and σ_d are the partition coefficients corresponding to the monomer and dimer endpoints, f_1 is the fraction of monomer, C_T is the total monomer concentration, and K_{dim} is the resolved equilibrium dissociation constant.

Partition coefficients obtained for molecular size standards were transformed as the inverse error function complement, $(\text{erfc})^{-1}(\sigma_w)$, to yield a linear relationship with the molecular Stokes radius (r) (Ackers, 1967):

$$r = a_o + b_o(\text{erfc})^{-1}(\sigma_w) \quad (4)$$

where a_o and b_o are calibration constants for a given column. Hydrated radii corresponding to the repressor monomer and dimer endpoints were calculated from calibration curves using molecular size standards α-chymotrypsin (22.4 Å), bovine serum albumin (35.5 Å), cytochrome *c* (16.4 Å), myoglobin (18.8 Å), and ovalbumin (27.3 Å). The linear relationship extended over a range of 16–35 Å. All calculations were performed on a Hewlett-Packard 9000 computer.

RESULTS

Genetic Screening of Repressor Mutants. Mutant cI repressors previously isolated were assayed for their ability to repress reporter gene expression from a synthetic operator/promoter (O_SP_S) and to confer immunity to superinfection by λ phage derivatives (Beckett et al., 1993); results are summarized in Table 1. O_SP_S consists of sites O_R1 and O_R2 (O_S) with a synthetic promoter, P_S, overlapping site O_S2; repression of downstream *cat* and *lacZ* synthesis increases with increasing occupancy of O_S2. Mutant and wild-type cI repressors are expressed from an IPTG-inducible *tac* promoter. Mutant repressor dimers harboring a defect in cooperative DNA binding will bind to O_S2 with greatly reduced affinity relative to a fully cooperative repressor due to unequal sharing of cooperative free energy in a two-site system (Ackers et al., 1983); alternatively, mutant repressors deficient in dimerization may fail to occupy the synthetic operator sites.

Of the eight proteins examined, six are extremely poor repressors of the synthetic promoter; cells containing these mutations are resistant to 20-fold higher levels of chloramphenicol than wild type and are as resistant as cells containing no repressor. The remaining two proteins exhibit approximately the same resistence as wild type. In the absence of IPTG, all of the constructs expressed minimal immunity to superinfection; however, upon induction, an increase in immunity spanning the full spectrum of values was observed. The level of phage immunity provides evidence for competent binding by the mutant proteins to superinfecting phage operator DNA. All but one of the proteins examined in this study (EK102) demonstrate reduced immunity relative to wild type in both the absence and the presence of IPTG, suggesting that they may be defective in some aspect of cooperative interactions at the level of either dimerization or cooperative binding to DNA. In the absence of specific defects in cooperative binding, immunity may reflect the relative concentration of binding species in the assay (Hecht & Sauer, 1985).

Dimerization Measurements. Large-zone elution profiles obtained for all of the mutant repressors displayed sharp boundaries and flat plateaus comparable in quality to those obtained for wild-type repressor (Beckett et al., 1990). Elution profiles for the leading edge of YH210 repressor are shown in Figure 2. The difference in elution volume between the monomeric and dimeric species is ~3 mL for YH210 as well as for wild type, EK102, GD147, and KN192, whereas this difference is only ~1.5 mL for SN228 and SR228. In addition, the elution volume for mutant repressor monomers was 0.2–0.6 mL smaller than that for wild type monomers and the elution volumes for mutant dimers were 0.6–1.3 mL smaller than that for wild type dimers. The implications of these dramatic changes in elution volume for hydrodynamic properties are discussed below. Weight average partition coefficients as a function of monomer concentration are shown in Figure 3 for the mutant repressors. Equilibrium dimerization constants resolved from the analysis are given in Table 2. Curves represent the best fits of the data to a monomer–dimer stoichiometric model of assembly. The dashed line in each panel represents wild-type assembly under identical solution conditions. The use of an assembly model that limits the stoichiometry to 2 is supported by two observations: (1) column calibration as a function of apparent molecular weight suggests that assemblies of three or more cI repressor monomers will exhibit a partition coefficient of less than 0.13, whereas the smallest values observed lie between 0.25 and 0.30; (2) sedimentation equilibrium ultracentrifugation data for repressor molecules which do not display mass action suggest a molecular weight that is consistent with the existence of dimeric repressor (see below). The data span 6 orders of magnitude in protein concentration and are well-described by the assembly model. EK102, GD147, KN192, and YH210 dimerize in the nanomolar range with essentially identical energetics, 0.1–0.5 kcal/mol weaker than wild type. In contrast, SN228 and SR228 dimerize considerably more weakly than wild type, by 3.5 and 2.2 kcal/mol, respectively.

Table 1: In Vivo Properties of Wild-Type and Mutant cI Repressors

repressor	phage immunity level[a] (−IPTG)	(+IPTG)	chloramphenicol resistance (μg/mL)
wild type	cIc17/vir	5v	10
EK102[b]	cIc17/vir	5v	20
SN198[b,c]	cIc17/vir	4v	20
EK188[c,d]	cIc17	3v/4v	200
YH210[d]	cIc17	3v/4v	200
GD147[b,e]	KH54	vir/3v	200
KN192[d]	KH54	vir/3v	200
SR228[d]	KH54	vir/3v	200
SN228[b]	KH54	cIc17/vir	200
no repressor[b]	KH54	KH54	200

[a] Relative virulence of bacteriophage: KH54 < cIc17 < vir < 3v < 4v < 5v. [b] Reported in Beckett et al. (1993). [c] Not examined in this study. [d] Isolated by Benson et al. (1994). [e] Isolated by Gimble and Sauer (1985, 1986).

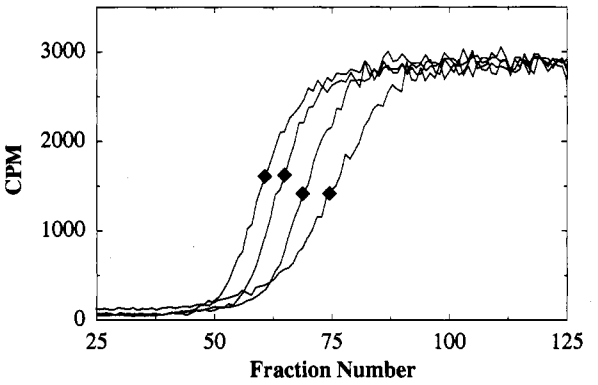

FIGURE 2: Leading edges of large-zone elution profiles for YH210 cI repressor. Total monomer concentrations increase from right to left (8.7×10^{-11}, 6.3×10^{-9}, 6.2×10^{-8}, and 1.2×10^{-5} M); (◆) the centroid (elution volume) for each zone.

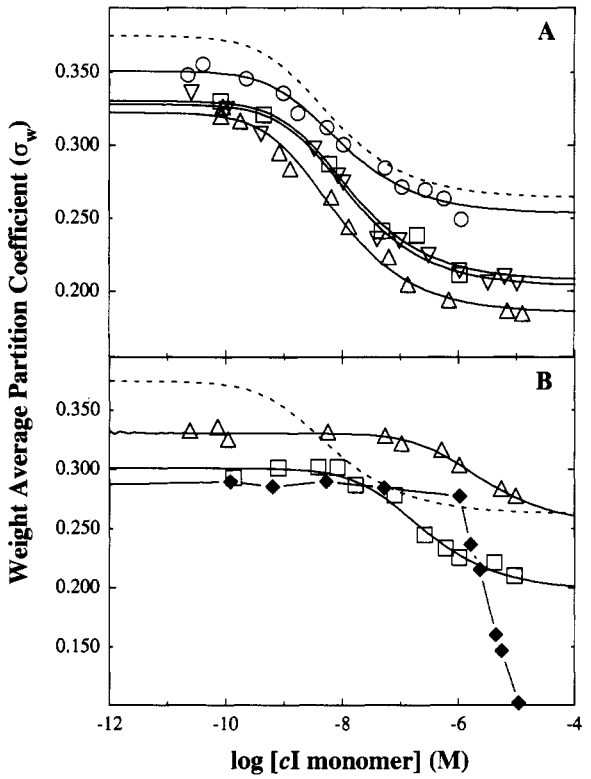

FIGURE 3: Weight average partition coefficients (σ_w) obtained as a function of total monomer concentration of mutant repressors. Curves represent best fits of the data to a monomer–dimer stoichiometry. Reference wild type association curves are shown as dotted curves. (A) (○) EK102; (▽) GD147; (□) KN192; (△) YH210. (B) (△) SN228; (□) SR228. (◆) PT158 reversibly aggregates to a stoichiometry greater than 2 (see text).

Table 2: Monomer–Dimer Equilibrium Constants of Wild-Type and Mutant *c*I Repressors

repressor	dimerization constant[a]	ΔG_{dim}[b]	σ[c]
wild type	1.8×10^8	−11.0 ± 0.3	0.008
EK102	1.1×10^8	−10.8 ± 0.3	0.005
GD147	7.5×10^7	−10.6 ± 0.3	0.007
KN192	7.3×10^7	−10.5 ± 0.5	0.007
YH210	1.3×10^8	−10.9 ± 0.2	0.006
SN228	3.7×10^5	−7.5 ± 0.2	0.004
SR228	3.9×10^6	−8.8 ± 0.3	0.006

[a] Association constant [*c*I monomer] (M^{-1}). [b] Standard Gibbs free energy in kcal/mol with 67% confidence intervals. [c] Square root of the variance of the fitted curve.

The apparent "flatness" of the unnormalized elution profiles for SN228 and SR228 presented in Figure 3 reflect differences in molecular shape and size relative to the other repressors examined. Since all data were fit to the same stoichiometry model, the shape of the fitted curve will be identical for normalized data, merely scaled along the concentration axis to the strength of the interaction. Normalization of the data via transformation of the concentration dependence of the partition coefficients into the fraction of monomeric species, f_1, was performed as a matter of course in fitting the data in order to resolve the dimerization free energy [see above; also Beckett et al. (1991)]; this transformation is not necessary for evaluation of hydrodynamic properties. An additional mutant repressor, PT158, was also examined for self-assembly. As seen from the chromatographic profile, PT158 remains monomeric up to a total concentration of ~1 μM, whereupon a precipitous decrease in partition coefficient is observed, by ~10 μM consistent with the formation of a species larger than the dimer. This higher order assembly is reminiscent of wild-type *c*I repressor, which has recently been shown to undergo such a transition in this same concentration regime (Senear et al., 1993). In our studies, data obtained at the highest concentrations (*ca.* 1–10 μM) for wild type and EK102 suggest that these repressors may be aggregating to a stoichiometry greater than 2. Concentrations greater than this are difficult to study chromatographically since prohibitively large amounts of material must be loaded on the column. Sedimentation equilibrium studies currently underway will facilitate assessment of the assembly state of the mutant repressors from ~1–100 μM total protein.

Table 3: Molecular Size and Shape Parameters for Wild-Type and Mutant *c*I Repressors

protein	species[a]	r (Å)[b]	r/r_o[c]	prolate (a/b),[e] w^d = 0.25	0.33	0.40	oblate (a/b),[e] w^d = 0.25	0.33	0.40
wild type	m	23.0	1.15	2.0	1.7	1.0	0.50	0.60	1.00
	d	28.7	1.14	2.0	1.7	1.0	0.50	0.70	1.00
EK102	m	25.0	1.25	3.5	3.0	2.6	0.30	0.35	0.40
	d	29.8	1.18	2.5	2.0	1.7	0.40	0.50	0.60
GD147	m	25.9	1.30	4.1	3.6	3.2	0.24	0.27	0.30
	d	31.6	1.25	3.5	3.0	2.6	0.30	0.35	0.40
PT158	m	26.9	1.35	4.8	4.4	4.0	0.20	0.23	0.25
KN192	m	26.4	1.32	4.3	3.9	3.5	0.23	0.25	0.28
	d	31.5	1.25	3.5	3.0	2.6	0.30	0.35	0.40
YH210	m	25.0	1.25	3.5	3.0	2.6	0.30	0.35	0.40
	d	31.5	1.25	3.5	3.0	2.6	0.30	0.35	0.40
SN228	m	26.0	1.30	4.1	3.6	3.2	0.24	0.27	0.30
	d	29.7	1.18	2.5	2.0	1.7	0.40	0.50	0.60
SR228	m	27.5	1.38	5.3	4.8	4.4	0.19	0.21	0.23
	d	31.8	1.26	3.6	3.1	2.8	0.28	0.32	0.35

[a] m = monomer; d = dimer. [b] Experimentally determined Stokes radii. [c] Ratio of Stokes radius to calculated anhydrous molecular radius. [d] Hydration (grams of H_2O per gram of protein). [e] Axial ratio for prolate and oblate ellipsoids.

Molecular Size and Shape Parameters. Partition coefficients resolved for the monomer and dimer endpoints of the association transition curve can be used, in conjunction with independent column calibration, to calculate Stokes radii for the monomeric and dimeric species; values are shown in Table 3. All mutations were found to have monomer radii 2–4.5 Å larger than wild type. In contrast, two size classes of mutant dimer are evident from the experimentally determined Stokes radii: EK102 and SN228 dimers are approximately 1 Å larger than wild-type dimer, while the remaining proteins, GD147, KN192, YH210, and SR228, exhibit essentially the same hydrated radius, ~2.8 Å larger than that of wild type. The inherent precision of molecular radius determinations of globular proteins by these gel partitioning methods has been shown to be ±0.2 Å (Warshaw & Ackers, 1971). The accuracy of the present experiments is estimated as ±0.3 Å, so the differences found are significant.

A lower limit for the molecular radius can be calculated *ab initio* for each species (monomer and dimer) by equating the anhydrous molecular volume $M\bar{V}/N$) to a sphere of radius r_o:

$$(M\bar{V}/N) = (^4/_3)\pi r_o^3 \quad (5)$$

Then

$$r_o = (3M\bar{V}/4\pi N)^{1/3} \quad (6)$$

where M is the molecular weight of the protein, $\bar{V}$ is its partial specific volume, and N is Avagadro's number. The hydrodynamic radius determined by analytical gel chromatography (Ackers, 1975) allows us to write the ratio of hydrated to anhydrous molecular radii as

$$r/r_o = r/(3M\bar{V}/4\pi N) = f/f_o \quad (7)$$

where f and f_o are the corresponding friction coefficients defined by Stokes' law:

$$f = 6\pi\eta r \quad (8a)$$

and

$$f_o = 6\pi\eta r_o \quad (8b)$$

for aqueous solutions of viscosity η. Calculating r_o^{mon} using M = 26 226 and $\bar{V}$ = 0.736 (calculated from amino acid composition) yields 20.0 Å for the anhydrous radius of wild-type cI repressor monomer. However, since r_{exp}^{mon} was found to be 23.0 Å, $(r/r_o)_{mon}$ = 1.15. Similarly, for wild-type dimers, r_o^{dim} = 25.2 Å and r_{exp}^{dim} = 28.7 Å; thus $(r/r_o)_{dim}$ = 1.14. Referring to Table 3, we note that four proteins exhibit a clear shift in r/r_o in the direction of compaction upon dimerization (EK102, KN192, SN228, and SR228). By contrast, wild type, YH210, and GD147 do not show this shift. The ratio r/r_o is identical for wild-type monomers and dimers, indicating similar molecular asymmetry/hydration for both species. However, all mutants display a larger value for r/r_o than wild type for both monomeric and dimeric species. The data therefore suggest mutation-induced expansion or partial unfolding in monomers, plus compaction upon dimerization. The latter reflects plasticity of the repressor which may be required for high-affinity binding to specific operator sites and for cooperative interactions when dimers are bound to adjacent sites on the tripartite right operator.

The frictional coefficient comprises two factors that reflect information regarding the macromolecular shape and the extent of hydration:

$$f/f_o = (f/f_o)_{shape}(f/f_o)_{hydration} \quad (9a)$$

$$= (f/f_o)_{shape}[1 + w/\bar{V}\rho_o]^{1/3} \quad (9b)$$

where w is hydration in grams of H_2O bound per gram of protein and ρ_o is the density of bound water. For a spherical molecule (a/b = 1) with r/r_o = 1.15 and w = 0.25 g of H_2O/(gram of protein), there are 364 mol of bound water/(mole of repressor). Plots of the frictional ratio, f/f_o (r/r_o), as a function of the ellipsoid axial ratio (prolate or oblate) *versus* hydration can be used to evaluate the asymmetry associated with each repressor species (Oncley, 1941). Table 3 shows the axial ratio predicted for prolate and oblate ellipsoids at three levels of hydration for each species examined in this study. A typical protein hydration of 0.25 g of water/(gram of protein) yields an axial ratio of 2:1 (prolate ellipsoid) or 0.5:1 (oblate ellipsoid) for wild-type repressor. A plausible explanation for the finding that monomers and dimers have identical asymmetry/hydration is that the "prolate" monomer assembles side to side resembling an "oblate" dimer. Values shown are consistent with an elongated molecular species as has been observed in sedimentation velocity experiments on wild-type repressor (Senear et al., 1993) and mutant cI repressors (this laboratory, unpublished experiments). These calculations also demonstrate that (1) all mutants have more expanded monomers than wild type, due perhaps to partial unfolding or extension of the linker between domains, and (2) EK102 and SN228 have the same dimer radius as wild type (r/r_o = 1.18); interestingly, these repressors exhibit full cooperativity, while the others exhibit a distinctly larger value for r/r_o (1.25–1.26) and pertain to species that are defective in cooperativity [Table 3 in Burz and Ackers (1994)]. Analysis of experimentally determined Stokes radii using frictional ratios facilitates interpretation of size data by consolidating both shape and hydration information from first principles.

DISCUSSION

An ongoing goal of our research program is elucidation of the molecular mechanism which gives rise to the cooperativity observed when cI repressor dimers bind to O_R, and evaluation of the importance of these cooperative interactions in the regulation of the life cycle of bacteriophage λ. The initial phase of the current study involved inception of a genetic screen to identify C-terminal domain single-site mutants of cI repressor with possible defects in cooperative interactions (Beckett et al., 1993). Three mutant repressors, EK102, GD147, and SN228, were obtained by random mutagenesis directed at the C-terminal region of the protein (Beckett et al., 1993), whereas the genetic screen presented in Benson et al. (1994) resulted in the isolation of spontaneous cooperativity mutants, EK188, KN192, YH210, and SR228, with amino acid replacement restricted exclusively to the C-terminal domain of the molecule. GD147 and PT158 were originally isolated as *ind* mutants by Gimble and Sauer (1985, 1986, 1989). Results of the genetic screen yielded proteins that failed to occupy a synthetic operator, presumably due to deficiencies in dimerization and/or cooperative binding, thereby allowing transcription of downstream reporter genes and survival of the cells against increasing levels of chloramphenicol. Immunity to superinfection, used to determine the relative binding avidity of mutant repressors, produced a spectrum of results that track closely with the measured equilibrium dimerization constant. Referring to Tables 1 and 2, reduced immunity is expressed concomitantly with weaker dimerization. This suggests that the immunity test is actually reporting the relative concentration of competent binding species, as observed by Hecht and Sauer (1985).

Since a cooperativity mutant candidate may be defective in its ability to dimerize and/or its cooperative binding to O_R, complete characterization requires the experimental determination of all free energy terms depicted in Figure 1. Analytical gel chromatography was used to measure the energetics of self-assembly for six of the eight candidates described in Table 1 as well as for PT158. Single-site replacement has very little effect on the dimerization properties of all but the position 228 mutants. Replacement of serine by asparagine or arginine significantly impedes the ability of the molecules to self-associate, weakening the energy of dimerization, relative to wild type, by 3.5 kcal/mol for SN228 and 2.2 kcal/mol for SR228. SN228 was independently isolated by Hochschild and Ptashne (1988), who observed that higher concentrations of SN228 were required to saturate a single operator site (O_R1) than for wild type. The authors took into account neither the activity of the mutant repressor, which was cited as being "relatively low" and "undetermined", nor the possibility that the protein was defective in its ability to dimerize. SN228, also isolated by Beckett et al. (1993), was found to bind less tightly to a single operator site (O_R1) as well as to tandem operator sites (O_R2/O_R3) when assayed using nitrocellulose filter binding. SN228 dimers have been shown to bind to specific sites in O_R with full cooperativity and no significant reduction in intrinsic free energy by quantitative DNAse I footprinting (Burz & Ackers, 1994). The explanation for the original observations lies in the inability of the molecule to form sufficient high-affinity binding species across the range of concentrations used in those experiments

due to a defect in dimerization, possibly coupled with a reduced activity of the protein. Serine 228 lies only eight residues from the C-terminus of the polypeptide chain. Terminal arms are known to affect association and binding in a number of systems; for example, the N-terminal "arms" of both *trp* and *c*I repressor are required for specific binding to operator DNA (Otwinowski et al., 1988; Pabo et al., 1982). Given the proximity of serine 228 to the terminus of the polypeptide chain, the structural perturbation resulting from replacement of that residue could disrupt protein–protein contacts between C-termini of monomers critical for dimer formation. Alternatively, the C-terminal region may mediate inter- or intradomain contacts necessary to maintain the correct monomer quaternary structure in order to dimerize. The structure of the C-terminal domain of *c*I repressor is not known, so that cocrystal structures of the N-terminal domain with O_L1 (Pabo et al., 1982; Jordan & Pabo, 1988) cannot provide insight as to the location or structural consequences of any of the mutations studied.

PT158 was originally isolated as an *ind*$^+$ mutation exhibiting greater susceptibility to *recA*-mediated cleavage (Gimble & Sauer, 1989). Degradation of *c*I repressor monomers constitutes an early step in the induction of lytic growth. The finding that PT158 is monomeric up to 1 μM is consistent with this observation. The PT158 mutation results in a selective loss of dimerization potential, but the mutant retains the ability to form higher order assemblies and bind to right operator DNA with wild-type cooperativity [Table 3 in Burz and Ackers (1994)]. Interestingly, and in contrast to properties observed for wild-type repressor, which forms octamers at concentrations above micromolar (Senear et al., 1993), GD147, KN192, YH210, SN228, and SR228 demonstrate no mass action beyond dimer molecular weight in preliminary sedimentation equilibrium experiments, whereas EK102, EK188, and PT158 do (unpublished experiments; T. Laue and S. Eden, personal communication). The "breakpoint" in the association curve for the mutants that undergo higher order assembly is about the same for wild-type repressor (Senear et al., 1993), suggesting a common mechanism for this process. Furthermore, the apparent decoupling of dimerization and higher oligomerization suggests that these properties are highly specific and may mediate functionally distinct processes in the life cycle of bacteriophage λ. Continued studies of the repressor will facilitate functional dissection of *c*I through separation of the two assembly pathways. The relationship between molecular size, state of assembly, and cooperative binding to O_R will be discussed elsewhere.

The change in Stokes radius arising from a single mutation is quite large and suggests considerable disruption of interactions required for maintenance of tertiary structure. It has been shown that interfacial amino acid replacement can result in both increases and decreases in the hydrodynamic radius of the mutated protein (Bromberg et al., 1989). Stokes radii determined from chromatography experiments indicate that all mutants exhibit a larger monomer radius than wild type; this increase in molecular size is not quantized but spans a range from 2 to 4.5 Å, corresponding to an increase of 10–20% in wild-type radius. The effective change in radius for mutant dimers is less severe, a reflection of the compacting effect of dimerization on the structure of the higher order assembly. With the exception of EK102 and SN228, mutant dimers are ~3 Å larger (10%) than wild type; radii calculated for dimers of EK102 and SN228 are about the same size as that of wild type. The larger dimer is observed only for the mutant repressors that are defective in cooperative operator binding, while the smaller EK102 and SN228 dimers are highly cooperative [Table 2 in Burz and Ackers (1994)]. The structural perturbations that accompany serine replacement at position 228 result in differential effects. The smaller SN228 dimer displays weaker assembly than SR228, yet it binds with wild-type cooperativity. This is consistent with the idea that the overall size that the high-affinity species presents to operator DNA is critical to cooperative binding; a 2.5-Å increase in diameter can render the system noncooperative. This could result from several factors. The change in repressor tertiary structure resulting from mutations could prevent intramolecular contacts required for cooperative binding by reorienting interacting residues. A larger species could experience steric hindrance upon operator binding that inhibits conformational changes required for formation of dimer–dimer contacts required for cooperativity. Alternatively, if *c*I binding alters the conformation of the DNA, larger dimers may preclude DNA conformational changes that would facilitate cooperative binding. The molecular diameter for wild-type dimers corresponds to a 17 base pair stretch of B-DNA; this is the size of an operator site. One can calculate that unless a significant amount of DNA bending occurs (*i.e.*, on the order of 30–35°), there is plenty of room for protein–protein contacts to be made within the volume limits calculated for mutant dimers; this suggests that steric hindrance may not be a factor. The discrete and separable functions of dimerization and higher assembly suggest a "sidedness" in the recognition processes that give rise to *c*I repressor function. The effects on molecular shape and size that have been inferred from the experiments described in this study could easily disrupt the alignment of surface structure necessary for self-association and cooperative DNA binding, underscoring the idea that global conformation is critical for correct function. Characterization of more cooperativity mutants will be required in order to shed light on the emerging patterns of association between molecular size and functional changes accompanying structural perturbation.

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