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A study of energetics of cooperative interaction using a mutant λ-repressor

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A λ-repressor mutant, S228N, which is defective in tetramer formation in the free state but retains full cooperativity, was studied in detail. Isolated single operator-bound S228N repressor shows association properties similar to those of the wild-type repressor. Fluorescence anisotropy studies with dansyl chloride-labeled repressor show a dimer–monomer dissociation constant of around 10^{-5} M. The structure of the mutant repressor was studied by circular dichroism, acrylamide quenching and sulfhydryl reactivity at protein concentrations of ≤10^{-6} M, where it is predominantly monomeric. The results suggest no significant perturbations in the structure of the S228N mutant repressor from that of the wild-type repressor. Urea denaturation studies also indicate no significant change in the stability of the repressor. The results were used to calculate energetics of loop formation in the cooperative binding process.

Keywords: cooperativity/free energy/loop/repressor/transcription

Introduction

Protein–protein interaction between two DNA-bound proteins is at the core of many important phenomena. This interaction was first demonstrated in DNA loop formation in *gal* and *ara* operon of *Escherichia coli* (Adhya, 1989; Schleif, 1992). Such protein–protein interactions form the basis of many other phenomena, including activation of transcription initiation. One recent proposal suggests that transient contact between polymerase and a regulatory protein is the key to understanding the activation process (Roy *et al.*, 1998).

Owing to the torsional rigidity of DNA, not all DNA-bound proteins are able to interact with each other. This has been clearly demonstrated in many systems where two otherwise interacting proteins are placed on the opposite faces of the DNA (Hochschild and Ptashne, 1986; Bandyopadhyay *et al.*, 1996). Clearly, the orientation of the interacting patches of the two proteins and the energy required to bring them to juxtaposition are crucial to interaction between the two proteins and loop formation. This concept is the basis of the differential contact model of transcription regulation (Roy *et al.*, 1998). Hence a more quantitative understanding of the energetics involved in protein–protein interaction while bound to DNA is essential.

Lytic–lysogenic switch of bacteriophage λ has emerged as one of the best characterized systems for the study of protein–protein interactions that regulate transcription (Ptashne, 1992). In this regulatory system, λ-repressor binds to several pairs of operator sites (e.g. O_R1–O_R2, O_L1–O_L2) with concomitant interaction between two adjacent site bound dimers. Primarily due to work of Ackers and co-workers (Senear *et al.*, 1986) we have obtained an estimate of the interaction energy, which is ~2–3 kcal/mol. This interaction energy is a net result of several distinct processes. In this work we have attempted to dissect the cooperative interaction into component processes which can then be related to structural changes. More importantly, we have attempted to estimate these various components of the interaction energy using a λ-repressor mutant, S228N, that is defective in higher order aggregate formation (Burz and Ackers, 1994; Burz *et al.*, 1994), but not in interaction between two dimers bound to adjacent operator sites.

Materials and methods

Materials

λc47 (cI$^-$) phage and pEA305 plasmid were obtained from Professors M.Lieb and M.Ptashne, respectively (Amman *et al.*, 1983). DTNB, acrylamide, IPTG, PMSF, BSA, polyethylenimine, DTT, calf thymus DNA, DNase and RNase, β-mercaptoethanol and glycerol were purchased from Sigma Chemical (St. Louis, MO). Restriction enzymes were obtained from GENIE (Bangalore India). Bacto-Agar, bacto-tryptone and yeast extract were supplied by Difco (Detroit, MI). All other reagents were of analytical grade and were procured from local suppliers.

Cloning of the cI gene of λ carrying c47 mutation

The *cIc47* mutant gene (same as S228N mutant repressor) of λ was cloned by ligating the 4.679 kb *Eco*RI/*Bam*HI fragment of λc47 DNA with the 3.987 kb *Eco*RI/*Bam*HI fragment of pBR322 to obtain the plasmid pMJ47. The *cI* gene in this plasmid was sequenced fully using suitably designed primers. A cassette plasmid pMS1 containing the wild-type *cI* gene under the control of tac promoter and having a *Cla*I site between the C-terminus of *cI* gene and the downstream *Hin*dIII site was constructed earlier (Das and Mandal, 1986). The S228N mutant *cI* gene was subcloned in pMS1 by exchanging its 803 bp DNA segment bounded by the C-terminal-upstream *Nsi*I and downstream *Cla*I sites with the same *Nsi*I–*Cla*I DNA fragment from the above pMJ47 to obtain pMSJ47 carrying the S228N mutant *cI* gene under the control of tac promoter.

Repressor purification

The S228N mutant repressor was purified from *E.coli* RR1 (Δ15 lacZ) carrying the plasmid pMSJ47 by the procedure given by Saha *et al.* (1992). The concentration of native repressor was determined using the relation $E_{1\%} = 11.3$ and was calculated in terms of monomer unless stated otherwise. For all studies, the repressor was dialyzed against 0.1 M phosphate buffer, pH 8.0.

Chemical modification

The repressor was dansyl labeled by treatment with a 10-fold molar excess of dansyl chloride according to Banik *et al.*

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(1993). Sulfhydryl reactivities were measured using DTNB as described by Banik *et al.* (1992).

Fluorescence methods

All fluorescence spectra were measured with a Hitachi F 3000 spectrofluorimeter with a computer for spectra addition and subtraction facility. For tryptophan fluorescence, the excitation and emission wavelengths were kept at 295 and 340 nm, respectively, and the inner filter effect correction was made according to Bandyopadhyay *et al.* (1995). Anisotropy was measured using a Hitachi polarization accessory as described by Banik *et al.* (1993). Acrylamide quenching methods were also described by Bandyopadhyay *et al.* (1995).

CD spectra

Far-UV circular dichroism (CD) spectra were measured in a JASCO J600 spectropolarimeter. The CD spectra were measured in a 1 or 10 mm pathlength cuvette as required, at ambient temperature, controlled at 25°C. The scan speed was 50 nm/min and 10 scans were signal averaged to increase the signal-to-noise ratio. This study was made in 0.1 M phosphate buffer, pH 8.0.

Denaturation study

A series of freshly prepared solutions of ultrapure urea having 0.5–10 M concentrations in 0.1 M potassium phosphate buffer (pH 8) were prepared and S228N repressor was added to a final concentration of 1 μM. Equal volumes of buffer were added to the same volume of urea solution and these mixtures were used as a blank. Tryptophan fluorescence intensity was monitored at 340 and 350 nm and the same experiment was repeated with wild type λ-repressor and compared.

Tetramer–dimer dissociation in the presence of O_R1

Dansyl-labeled S228N repressor was mixed with unlabeled S228N repressor at a ratio of 1:9 to achieve a high concentration (~30 μM). It was then mixed with a stoichiometric amount of single operator O_R1 so that final concentration of the operator was 15 μM, to form operator-bound S228N complex. The anisotropy value of this complex was measured. It was then progressively diluted with 0.1 M potassium phosphate buffer, pH 8.0, and anisotropy values at different protein concentrations were determined. In all cases the buffer blank value was subtracted from each experimental value. The excitation wavelength was 340 nm and the emission wavelength was 520 nm. A computer averaging transient scan was performed to increase the signal-to-noise ratio.

Curve fitting

Curve fittings were carried out with Kyplot freeware (Koichi Yoshioka, 1997–99; version 2.0, beta 4, 32 bit). The following equation was used to fit monomer–dimer and O_R1 complex data, as the latter data represent the association between two molecules of preformed complex and formally equivalent to monomer–dimer equilibrium:

$$A_{obs} = A_d + \frac{(A_m - A_d)[(-1 + \sqrt{(1 + 8[P]/K_d)}]}{4[P]/K_d}$$

where A_{obs} is the observed anisotropy, A_m is the anisotropy of the monomer, A_d is the anisotropy of the dimer, K_d is the dissociation constant and [P] is the total monomer concentration.

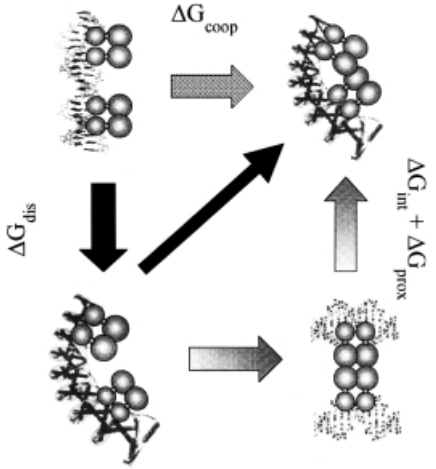

Fig. 1. Binding cycle of the cooperative binding process of λ-repressor to adjacent pairs of operator sites. The upper left-hand corner is the complex with two repressor dimers bound to operator sites without cooperative contact, the lower left-hand corner is the hypothetical distorted complex without protein–protein contact, the lower right-hand side complex indicates a complex between two repressor dimers on isolated operator sites and the upper right-hand corner is the fully formed cooperative complex. A negative sign before free energy indicates favorable interaction.

Results

Estimation of interaction energy

The λ-repressor dimer binds to pairs of operator sites with concomitant interaction between the dimers resulting in cooperative binding. This cooperatively bound complex is accompanied by DNA and protein distortion. Figure 1 shows the deconstruction of the cooperative complex formation in terms of steps that can be estimated and related to structural changes. Clearly, the net cooperative interaction energy (ΔG_{coop}) is sum of the interaction energy between two repressor dimers bound to two isolated operators (ΔG_{int}), loss of rotational and translational entropy by being associated with the same DNA molecule (as opposed to two separate DNA molecules) (ΔG_{prox}) and the protein–DNA distortion energy needed to bring the two interacting protein surfaces in juxtaposition (ΔG_{dis}):

$$\Delta G_{coop} \approx \Delta G_{int} + \Delta G_{prox} + \Delta G_{dis}$$

From an experimental point of view, the exact equivalence of the two sides is not possible as ΔG_{int} can only be measured for two repressors bound to identical operator sites and not to different operator sites, e.g. O_R1 and O_R3. As has been mentioned above, ΔG_{coop} has been estimated for λ-repressor cooperative interaction. Thus, measurement of any two quantities on the right-hand side of the equation should be sufficient to arrive at an estimate of all three quantities. Both ΔG_{prox} and ΔG_{dis} are difficult to measure experimentally. As will be described below, however, the magnitude of ΔG_{prox} can be estimated based on experiments done on other systems. Thus an estimate of ΔG_{int} should provide an estimate of energies of steps leading to cooperative complex formation.

We have already reported a method for measuring the interaction energy of two repressor dimers bound to an oligonucleotide containing an operator site (Banik *et al.*, 1993). Owing to operator-induced conformational changes in the repressor and consequent modulation of the nature of the cooperative interaction energy (Bandyopadhyay *et al.*, 1996), it is necessary that the interaction energy be measured between repressors bound to isolated operator sites. However, such a

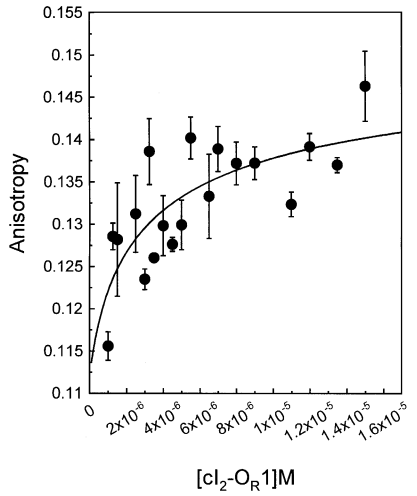

Fig. 2. Fluorescence anisotropy of dansyl-labeled S228N repressor–O_R1 complex as a function of complex concentration. The repressor was dansyl labeled as described in the Materials and methods section. The labeled repressor was mixed with an oligonucleotide containing a sequence of O_R1 in 1:1 dimer to repressor stoichiometry. The complex was serially diluted with buffer and fluorescence anisotropy measured. The buffer conditions were 0.1 M potassium phosphate buffer, pH 8.0. The temperature was 25°C. The excitation wavelength was 340 nm and the emission wavelength was 520 nm.

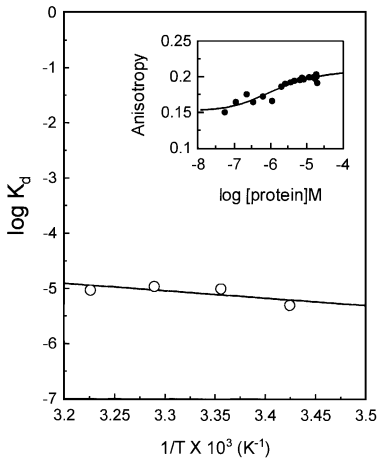

Fig. 3. Van't Hoff plot of dimer–monomer dissociation of S228N repressor. The dissociations were determined in 0.1 M potassium phosphate buffer, pH 8.0, at 19, 25, 31 and 37°C. The inset shows the anisotropy vs repressor concentration plot at 19°C.

measure of interaction energy cannot be taken as a measure of ΔG_{int} without additional evidence. It needs to be established that the interface between the two isolated operator-bound repressors is the same as the interface involved in the cooperative interaction.

Ackers and co-workers have demonstrated that S228N mutant of λ-repressor retains full cooperative interaction energy, but at the same time loses much of the ability to form free tetramers in solution (Burz and Ackers, 1994; Burz *et al.*, 1994). In addition, S228N mutant is defective in monomer–dimer association (see later) (Burz and Ackers, 1994). Despite the weakened monomer–dimer association, the binding of S228N to the O_R1 operator site is complete below 1 μM protein concentration (Burz and Ackers, 1994). The dimer–operator interaction energy is sufficient to overcome the monomer–dimer association defect in this concentration range. Hence it is possible to measure the interaction between two S228N dimers complexed with operator site O_R1 by measuring self-association of the complex at concentrations above 1 μM. Figure 2 shows the fluorescence anisotropy of dansyl-labeled S228N λ-repressor–O_R1 complex as a function of complex concentration. The anisotropy increases as a function of complex concentration, suggesting association between two dimers. A fit to the appropriate equation (see Materials and methods) yields a dissociation constant of 6 μM. Thus, although this mutant has lost its ability to tetramerize in the free state, like the cooperative interaction, it has largely retained the interacting ability while operator bound. This strongly suggests that the interface involved in the cooperative interaction is similar to, if not identical with, that of the repressors bound to two isolated operator sites.

To check whether dansylation has any untoward effect on the repressor structure, we determined the dimer–monomer dissociation constant at various temperatures. The inset in Figure 3 shows the values of anisotropy at various repressor concentrations at 19°C. This was fitted to obtain the dissociation constants. Figure 3 shows the van't Hoff plot ($\ln K_d$ vs $1/T$). The plot is nearly parallel to the $1/T$ axis, indicating a near zero change in ΔH. The monomer–monomer association in this mutant repressor is thus entropy driven. In a recent study, Burz *et al.* (1994) obtained similar results for unmodified dimer–monomer dissociation of the S228N repressor, suggesting no major change resulting from chemical modification. The wild-type van't Hoff enthalpy was estimated by Burz and Ackers (1996) to be ~–15 kcal/mol. Clearly, such a major change in thermodynamic parameters indicates a substantial reorganization of the monomer–monomer interface. How such a relatively conservative substitution leads to such a major reorganization is an unresolved question. This is particularly so since the mutation does not cause any major structural change in the monomer (see later).

Structural integrity of S228N repressor

The similar association of properties of S228N repressor while bound to a single operator site suggests that such an interaction energy may be interpreted as ΔG_{int}. It would be wise, however, to make such an interpretation only if the structure of the mutant repressor remains similar to that of the wild-type. Mutations are known that cause phenotypic changes indirectly by causing structural perturbations (Deb *et al.*, 1998). Such structural perturbations may lead to new interaction surfaces unrelated to the native one. We therefore explored the structure of the S228N repressor with various spectroscopic and biochemical tools. Figure 4A shows that the CD spectra of both wild-type and the S228N repressor at 0.5 μM concentration are virtually identical. Since, at this concentration, the wild-type protein is dimeric and the S228N mutant repressor is monomeric, the above result suggests that very little change occurs in the secondary structure following dimer formation. Figure 4(B) shows the CD spectra of both the wild-type and S228N repressors at 15 μM concentration. At this higher concentration, the wild-type repressor is predominantly in the tetrameric state (Banik *et al.*, 1993), whereas the S228N mutant repressor is present in the dimeric state along with a significant fraction of the population in the monomeric form (Burz and Ackers, 1996). Hence the identical nature of the spectra of the two repressors even at a concentration as high as 15 μM also suggests very little change in secondary structure following tetramer formation by the wild-type repressor. Clearly the

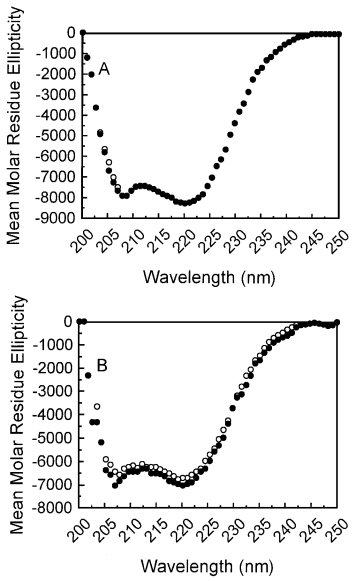

Fig. 4. CD spectra of S228N mutant (●) and wild-type repressors (○). Spectra were measured in 0.1 M potassium phosphate buffer, pH 8.0, at 25°C at protein concentrations of (A) 15 and (B) 0.5 μM. The small difference in molar mean residue ellipticity between the two concentrations is probably due to pipetting error.

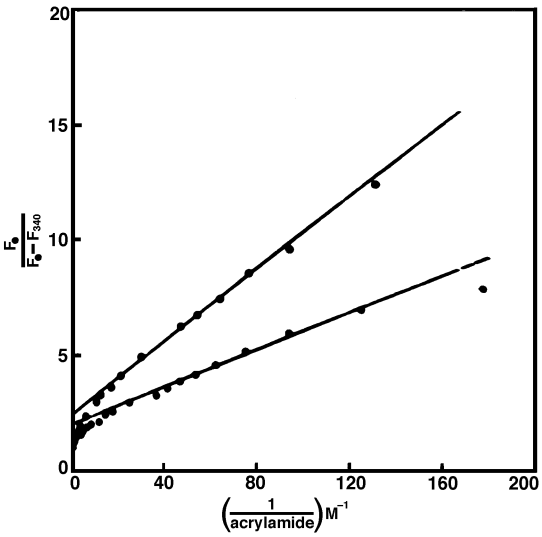

Fig. 5. Lehrer plots of acrylamide quenching of S228N mutant repressor. The spectra were measured in 0.1 M potassium phosphate buffer, pH 8.0, at 25°C. The excitation and emission wavelengths were 295 and 340 nm, respectively. Each point is an average of four independent determinations. The repressor concentrations used were 0.5 μM (○) and 20 μM (●).

secondary structure of S228N repressor is very similar to, if not identical with, that of the wild-type.

Another way of judging the conformation of the C-terminal domain and the hinge region is to use collisional quenching of tryptophan fluorescence. The λ-repressor has three tryptophan residues: one within the hinge region at position 129 and two in the C-terminal domain at positions 142 and 230. Acrylamide quenching of tryptophan fluorescence has been effectively used to separate different quenchable components and has proven effective in monitoring conformational changes in λ-repressor (Bandyopadhyay *et al.*, 1995). Figure 5 shows the Lehrer plots of acrylamide quenching of tryptophan fluorescence of S228N mutant repressor at 0.5 and 20 μM concentrations of the protein. From the monomer–dimer dissociation data obtained by fluorescence anisotropy (Figure 3), it is clear that the above mutant repressor will be predominantly monomeric at 0.5 μM and dimeric at 20 μM concentration. The Lehrer plot in these two instances cuts the *y*-axis at around 2. The most quenchable parts were calculated to have K_{sv}s of 42.5 M^{-1} at 0.5 μM and 28.7 M^{-1} at 20 μM This difference appears to be very modest. This suggests that only a small change in quenchablities of the tryptophan residues occurs upon monomer formation. Most importantly, the Lehrer plot of S228N repressor is not unlike that of the wild-type protein under dimeric conditions, suggesting preservation of the tertiary structure (Bandyopadhyay *et al.*, 1995).

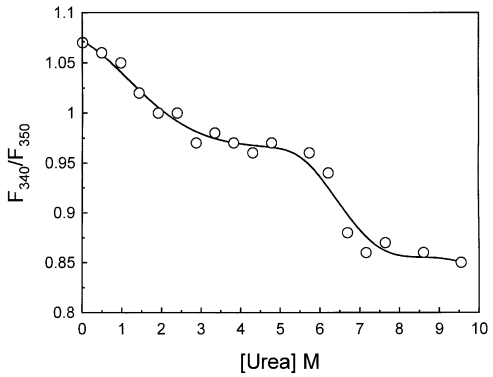

Fig. 6. Urea denaturation profile of S228N repressor. The study was conducted in 0.1 M potassium phosphate buffer, pH 8, and the S228N repressor concentration was 1 μM. The excitation wavelength was 295 nm. The line was drawn so as to obtain the best visual fit.

Wild-type λ-repressor has three cysteine residues, all of which are in the C-terminal domain and in the native repressor none of them is reactive towards sulfhydryl reagents such as DTNB. The sulfhydryls are resistant to the above reaction even in 6 M urea. Only in 9 M urea does one sulfhydryl group become reactive to DTNB, suggesting a very stable core in the wild-type protein (Banik *et al.*, 1992). In order to see if the S228N mutation had destabilized this core, we carried out the DTNB reaction of this mutant repressor at 25°C at 10 μM concentration. Neither the wild-type nor the S228N repressor shows any significant sulfhydryl reactivity (0.004 and 0.002 mol/mol –SH groups reacted, respectively), which suggests that the above mutation has little effect on the C-terminal domain stability. Since at 10 μM concentration more than 50% of the population of S228N repressor is in the monomeric form, the above results suggest that none of the sulfhydryl groups are at the monomer–monomer interface.

Another way to measure destabilization of a protein by mutations is through measurement of denaturation. Figure 6 shows the effect of urea concentration on the F_{340}/F_{350} ratio of S228N γ-repressor. As the urea concentration increases from 0 to 10 M, there is a progressive shift of the emission maximum towards the red. The wild-type repressor shows two distinct transitions centered around 2 and 6.5 M urea (Deb *et al.*, 1998). In the case of S228N, the nature of urea denaturation is identical with that of the native repressor, i.e. the low urea transition occurs at 1.9 M urea and the high urea transition at 6.7 M urea. In the native S228N repressor, the F_{340}/F_{350} ratio starts around 1.07 and levels off around 0.86 with an intermediate having an F_{340}/F_{350} ratio of ~0.97, identical with that of the wild-type repressor under the same conditions. In 6.7 M urea, the emission maximum has shifted to 352.8 nm.

In 9.6 M urea the emission maximum has shifted to 353.6 nm and the intensity at 340 nm has fallen to about 59.8% of that of the S228N repressor in the absence of urea. Hence, from the denaturation experiment, we conclude that the general integrity of the C-terminal domain structure is preserved in the mutant repressor.

Discussion

S228N is an interesting mutation, since this is the only mutation known to affect free dimer–tetramer association, while leaving cooperativity intact. Probing the three-dimensional structure of the mutant protein with various techniques suggests that the tertiary and secondary structures are not significantly affected by the mutation. This argues for the notion that the effects of the mutation are direct and the residue S228 is directly involved in free dimer–tetramer association but not in cooperativity. Since free dimer–tetramer association in solution is significantly weakened without affecting isolated operator-bound association, it is very likely that the interaction interface in the latter situation is very similar to, if not identical with, the cooperative interaction interface. Thus isolated operator-bound interaction energy is a true measure of ΔG_{int}. It is an interesting question as to how a relatively conservative substitution can cause very significant weakening of monomer–monomer interaction. One possible mechanism is steric. However, S228R (which has a bulkier side-chain) has less monomer–dimer defect (Burz and Ackers, 1996), suggesting that steric clash is not the sole reason. Another possible mechanism may be that the β-hydroxyl group of serine forms a hydrogen bond with a partner, which is disrupted in arginine or asparagine substitution.

If ΔG_{coop} is –2 to –3 kcal/mol and ΔG_{int} is ~–8 kcal/mol (obtained from measurement of self-association of dimer–operator complexes), then the sum of ΔG_{dis} and ΔG_{prox} should be about 6–7 kcal/mol (negative sign indicating favored towards loop formation). Although we cannot measure ΔG_{prox} directly, we can estimate its magnitude. One possible way is to look at the effect of fusing two protein molecules on some properties that occur *in trans*. Such an approach was taken by Robinson and Sauer (1996a,b). In their study, and also other studies summarized in their papers, the effective concentration [K_d(bimolecular)/K_d(unimolecular) ratio] values are relatively small, ~3×10^{-3} M. Based on their results, we estimate that ΔG_{prox} is not very large. Hence loss of rotational and translational entropy upon binding to a piece of DNA does not contribute a great deal of free energy. This is not unanticipated since the only additional loss involved is loss of three degrees of rotational and three degrees of translational freedom ($3RT \approx 1.8$ kcal/mol) (this is on the assumption that no preferential orientation occurs on the DNA). Hence ΔG_{dis} is likely to be ~8 kcal/mol with a lower limit of ~6 kcal/mol (i.e. when ΔG_{prox} is zero). Even the lower value of ΔG_{dis} indicates that a very significant DNA and/or protein distortion is present, consistent with CD and fluorescence studies (Roy *et al.*, 1998).

It is interesting that when the two operators were placed such that their separation is around 5–6 turns of DNA, the cooperative interaction occurred. If the protein–protein interface in such a case is similar to the natural scenario, it would imply that the distortion to generate a loop of that size would be ~6–8 kcal/mol. This value is not inconsistent with the theoretical estimate (Hochschild and Ptashne, 1986).

The energetics of loop formation have a direct bearing on the mechanism of transcription activation and repression. In the differential contact model, the ability of the regulator to contact the appropriate intermediate or the transition state is crucial to the outcome, i.e. repression or activation. This ability to establish contact transiently is in turn dependent on favorable energies of loop formation during that time period. An understanding of the underlying energetics is therefore crucial for a mechanistic understanding of the process of activation and contact repression.

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