Flanking DNA-sequences contribute to the specific binding of *cI*-repressor and O_R1

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ABSTRACT

The binding of *cI*-repressor to a series of mutant operators containing O_R1 of the right operator of bacteriophage lambda was investigated. Sites O_R2 and/or O_R3 were inactivated by either point or deletion mutations. The free energy of binding repressor to O_R1 in the wildtype operator, ΔG_1 , is -13.7 ± 0.3 kcal/mol. ΔG_1 determined for an O_R2^- operator created by a single point mutation in O_R2 is -13.6 ± 0.2 kcal/mol. In contrast, ΔG_1 for the binding of repressor to a cloned synthetic O_R1 operator containing only 24 bp of lambda sequence is -12.2 ± 0.1 kcal/mol. When sequence 5' to O_R1 is present, the binding affinity increases to -13.0 ± 0.1 kcal/mol. In addition, the proximity of O_R1 to a fragment-end decreases ΔG_1 from -13.7 to -12.3 ± 0.1 kcal/mol. These results suggest that the DNA sequence outside the 17 bp O_R1 binding-site contributes to the specific binding of *cI*-repressor.

INTRODUCTION

The regulation of both prokaryotic and eukaryotic gene transcription depends on interactions between specific control regions of DNA and regulatory proteins (c.f. 1,2). The specific binding-sites of regulatory proteins are generally identified by the ability of a protein to protect a region of DNA-sequence from chemical or enzymatic modification or degradation (3-6). The analysis of mutations which inhibit protein binding is often used to infer the critical contacts within the protected region necessary for a protein to recognize a specific DNA-sequence. Additional evidence that the basepairs which mediate site-specific DNA recognition are located exclusively within binding-sites defined by protection methods comes from models of the docking of DNA-binding proteins with specific DNA-sequences and, more recently, from x-ray diffraction studies of co-crystals of proteins and their specific DNA binding-sites (7-10).

Mutation analysis and crystallographic studies focus on the protected regions and in general have not explicitly considered the DNA flanking the binding-sites. Thus, *attenuation* of specific binding by elements outside the protected region might be overlooked. To order to assess this possibility, quantitative footprint titration studies of the binding of *cI*-repressor ('repressor') to site O_R1 of the three-site, wildtype lambda right operator (O_R) and to O_R1 of O_R2^- and $O_R2^-3^-$ mutant operators were conducted. The DNA context of O_R1 in the mutants ranged from a single basepair substitution in O_R2 to complete deletion of the lambda sequence both 3' and 5' to O_R1 . The ability of the footprint titration method to measure *individual-site* binding curves for each site allows us to directly compare the *intrinsic* affinity of repressor for O_R1 even in the presence of other competent sites. In this paper, we demonstrate that both the presence, and the sequence, of the DNA flanking

O_R1 has a significant effect on the binding of repressor to the site. This dependence of *intrinsic* binding free energy of a DNA-binding protein on the sequence context suggests an additional level of complexity in the mechanism of DNA-sequence recognition by proteins.

EXPERIMENTAL

Materials. All reagents were reagent grade obtained from commercial sources and used without further purification. Restriction enzymes were from either Bethesda Research Labs or New England Biolabs. Klenow fragment was from New England Biolabs. ³²P-labeled deoxynucleotides of 3000 Ci/mmol activity were obtained from Amersham. Preparation and radiolabeling of restriction fragments was by standard procedures (11) and has been described previously (12). Plasmid pKB252 (13) and pBJ306 (14) were gifts from M. Ptashne and J. Ellison, plasmid pOR1 (15) was a gift from R. Sauer and H. Nelson, and plasmid pAB101 (16) was a gift from A.D. Barone. The position of restriction sites within regions of lambda DNA sequence are indicated by the number following each enzyme name (17). Plasmid pKB252 contains the wildtype lambda right operator. Plasmid pBJ306 was derived from pKB252 and differs by a single basepair substitution in O_R2. Restriction fragments were excised from these plasmids by digestion with Bgl II (38103) and Hind III (37584) to yield 648 bp fragments. Plasmid pOR1 containing only site O_R1 had been constructed (15) from the wildtype operator by deletion of the DNA sequence downstream of the Hinc II site in O_R2 (37989) followed by ligation to the vector with Eco RI linkers. Two different restriction fragments containing O_R1 were generated from this plasmid. A 155 bp O_R1 containing fragment with the ³²P-labeled end 15 bp from O_R1 was generated by cleavage of with Eco RI and Hae III (38187). A restriction fragment of 2800 bp with the ³²P labeled Bgl II end 93 bp from O_R1 was generated by cutting with Bgl II (38103) and Ava I. Plasmid pAB101 is pUC8 (18) with a 23 bp oligonucleotide cloned into the SmaI site in the polylinker (16). A restriction fragment 300 bp long with the labeled end 120 bp from the synthetic O_R1 operator was generated by digestion of plasmid pAB101 with Ban I and Bgl I.

Methods. The protocols for the quantitative DNase footprint titration assays which were conducted have been described in detail elsewhere (12). All the equilibrium binding experiments were conducted in a buffer consisting of 10 mM Bis-tris, 2.5 mM MgCl₂, 1mM CaCl₂, 0.1 mM EDTA, 200 mM KCl, 100 mg/mL BSA, and 2 μg/mL sonicated calf thymus DNA at pH 7.00 ± 0.01 and 20 ± 0.1°C. Equilibrium mixtures contained repressor concentrations as indicated and labeled operator DNA such that the chemical concentration of the DNA was less than the dissociation constant of repressor dimer for the tightest binding-site of the particular operator. Since the repressor dimer concentration is effectively buffered by the large excess of monomers with which it is in equilibrium it is unnecessary to correct for the total operator DNA concentration, which is not accurately known. Thus, we can make the assumption in our determination of binding constants that free protein is equal to total protein.

The purification of the repressor used in these studies has been described previously (12). The repressor is greater than 95% pure with a specific DNA-binding activity of 79%. Total monomer concentrations were corrected for this activity prior to calculation of the dimer concentration. Repressor dimer concentrations were calculated from the K_d measured at 0°C, pH 8.0, 200 mM KCl (19). The assumption is made that the dimerization of repressor is unchanged at our conditions.

Table I: Free Energies Resolved from Individual-site Isotherms of the Wildtype Operator (kcal/mol).*

ΔG_1	=	-13.7 ± 0.3
ΔG_2	=	-10.3 ± 1.9
ΔG_3	=	-10.2 ± 0.5
ΔG_{12}	=	-3.6 ± 1.9
ΔG_{23}	=	-2.9 ± 2.0
$s^\#$	=	0.082

*Simultaneous analysis of the individual-site binding curves from repressor binding to wildtype, O_R1^- , and O_R3^- operators. Data are taken from reference 23.

[#]Square root of the variance of the fitted individual-site binding curves.

Data Analysis. Individual data-sets were analyzed (with the endpoints as adjustable parameters) according to <eq. 1> using non-linear least squares procedures to estimate the binding free energies and their 65% confidence intervals (12).

RESULTS

Wildtype O_R . In order to consider the effect of the DNA sequence flanking O_R1 on repressor binding, we first consider the *intrinsic* free energy of binding, ΔG_1 , in the wildtype operator. Repressor binding to O_R is characterized by three *intrinsic* binding free energies ΔG_1 , ΔG_2 , and ΔG_3 , and two additional free energies, ΔG_{12} and ΔG_{23} , which account for pairwise *cooperative* interactions (20). The intrinsic binding free energies describe repressor binding to each site in the absence of repressor-binding to other sites. These energies were obtained by comparison of the *individual-site binding curves* (21) for repressor binding of repressor to the wildtype operator and to mutant operators ('reduced-valency') in which one or more sites are rendered incapable of repressor binding (Table I). This analysis dissects the repressor- O_R interactions into the separate binding and cooperative processes, and yields the Gibbs interaction energy for each. A value of ΔG_1 of -13.7 kcal/mol was obtained for the wildtype operator under these experimental conditions (see Materials and Methods). Detailed discussions of this analysis procedure have been published (22,23).

O_{R2}^- Operator. O_{R1} containing DNA-operators in which O_{R2} was inactivated by a single-basepair substitution, and in which both O_{R2} and O_{R3} were removed by sequence deletion were studied. The currently accepted model for cooperative binding of repressor to O_R suggests that only pairwise interactions between repressor molecules bound to adjacent operator sites, i.e. between O_{R1} and O_{R2} and between O_{R2} and O_{R3} , exist (1,20). Therefore, the smallest perturbation in operator DNA-sequence which would result in non-cooperative binding of repressor to O_{R1} is an operator which contains a single basepair substitution in O_{R2} (Fig. 2a) that eliminates binding of repressor (O_{R2}^-). The individual-site binding curves for repressor binding to *each* site of the O_{R2}^- operator were determined. No binding to O_{R2} was detected over the repressor concentration range analyzed (data not shown).

The binding data for O_{R1} and O_{R3} were analyzed according to a model which considers cooperative interactions between sites. The results of this analysis preclude significant cooperative interaction between repressor molecules bound at O_{R1} and O_{R3} (manuscript in preparation). Thus, the individual-site binding data for O_{R1} (Fig. 1) in the O_{R2}^- operator were analyzed as a single independent site in which binding is described by the

Figure 1. Individual-site binding curves for O_R1 from 4 different operators. *Top panel:* Triangles, $O_R2^{-3^{-}}$ operator from pOR1 in the Bgl II / Ava I restriction fragment (Table II, B2; Fig. 3b). Squares, $O_R2^{-3^{-}}$ operator from pOR1 in the Eco RI / Hae III restriction fragment (Table II, B1; Fig. 3b). *Bottom panel:* Squares, $O_R2^{-3^{-}}$ operator (Table II, A; Fig. 3a). The titration of O_R3 has been omitted for clarity; Triangles, $O_R2^{-3^{-}}$ operator created by oligonucleotide synthesis (Table II, C, Fig. 3c). The binding curves shown by the solid lines are calculated for the best-fit value of ΔG_1 listed in Table II.

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$$Y_1 = \frac{K_1[X]}{1 + K_1[X]} \quad (1)$$

where K_1 is the equilibrium association constant (related to the free energy by $\Delta G_1 = -RT \ln K_1$) and $[X]$ is the free ligand concentration. Fitting of this expression by numerical analysis to the individual-site binding curves determined for the mutant operators shown in Fig. 1 was used to determine ΔG_1 . ΔG_1 obtained in this manner is within experimental error of the value measured for the wildtype operator (Table II). The introduction of this particular single-basepair substitution in O_R2 apparently does not

Table II: Free Energies Resolved from the Binding Curves of the Different $O_R2^{-3^{-}}$ Operators (kcal/mol).

Operator	Restriction fragment	# expts	ΔG_1	s
O_R2^{-} (A)		4	$-13.6 \pm .2$	0.078
$O_R2^{-3^{-}}$ (B1)		3	$-12.3 \pm .1$	0.058
$O_R2^{-3^{-}}$ (B2)		3	$-13.0 \pm .1$	0.029
$O_R2^{-3^{-}}$ (C)		2	$-12.2 \pm .1$	0.051

Free Energies resolved from the binding curves shown in Fig. 1. (A) 1102bp Bgl II / Pst I restriction fragment from pBJ306. O_R1 is located 93 bp from the labeled Bgl II site. (B1) 155bp Eco RI / Hae III restriction fragment from pOR1. O_R1 is located 15 bp from the labeled Eco RI site. (B2) 2800bp Bgl II / Ava I fragment from pOR1. O_R1 is located 93 bp from the labeled Bgl II site. (C) O_R1 within 22bp of lambda sequence from pAB101. The Ban I / Bgl I restriction fragment is 300 bp. O_R1 is located 100 bp from the labeled Ban I site. Thin and thick lines indicate lambda and non-lambda DNA, respectively.

Figure 2. The sequence of lambda DNA in the region of the O_R operator. Boldface capital letters indicate O_R binding-sites (17), lower case letters indicate lambda sequence outside the binding-sites and underlined lower case letters indicate non-lambda sequence from the vectors into which the O_R control region was cloned. **A.** Wildtype phage lambda sequence. The single base-pair substitution of the reduced-valency mutant O_R2^- is shown underneath. **B.** The $O_R2^-3^-$ deletion mutant pORI. Lambda DNA sequence ends 7bp upstream from O_R1 . **C.** $O_R2^-3^-$ operator (pAB101) constructed from a 23 bp synthetic oligonucleotide containing O_R1 cloned into the Φ Small site of pUC8.

O_R2⁻³⁻ Operators. In contrast to this result, the values of ΔG_1 determined for operators which lack portions of the lambda sequence flanking O_R are significantly different. O_R2^{-3-} operators lacking either lambda sequence 5' of O_{R1} or lambda sequence both 3' and 5' of O_{R1} were analyzed. In plasmid pOR1 the sequence 5' of O_{R1} is deleted, eliminating sites O_{R2} and O_{R3} (Fig. 2b). In addition, the Eco RI / Hae III restriction fragment places O_{R1} 15 bp from the ^{32}P labeled end (Fig. 3b). As seen in Table II, ΔG_1 determined for this fragment is 1.4 kcal/mole more positive than ΔG_1 resolved from the wildtype and O_{R2}^{-} operators (Fig. 1).

The figure displays three restriction enzyme maps, labeled A, B, and C, representing the 3' ends of the human and mouse $\alpha 1(I)$ procollagen genes. Each map shows the positions of specific restriction sites and the resulting fragment sizes.

- Map A:** Shows the human gene. Restriction sites include *Hae*III (38137), *Bgl*II (38103), *Hinc*II (37988), *Hind*III (37584), and *Pst*I (37005). Two fragments are indicated: *Bgl*II (648bp) and *Bgl*II (1102bp).
- Map B:** Shows the mouse gene. Restriction sites include *Eco*R1, *Hinf*I (38394), *Hae*III (38137), *Bgl*II (38103), *Eco*R1, and *Ava*I. Two fragments are indicated: *Eco*R1 (155bp) and *Bgl*II (2800bp).
- Map C:** Shows the human gene. Restriction sites include *Ban*I and *Bgl*I. One fragment is indicated: *Ban*I (300bp).

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effects, an alternate set of restriction cuts (Bgl II / Ava I, Fig. 3b) was used to generate a fragment end further from O_R1 . The value of ΔG_1 resolved from this fragment is still 0.7 kcal/mol more positive than that resolved for the wildtype operator, but 0.8 kcal/mol less positive than that obtained for the Eco RI / Hae III restriction fragment (Fig. 1: Table II). Thus, the lambda sequence 5' of O_R1 contributes to the specific binding of repressor. Even a non-specific DNA sequence can contribute to binding of the operator, since the absence of DNA (the result of the proximity of O_R1 to a fragment end) causes a further decrease in binding affinity.

The binding of repressor to a cloned synthetic O_R1 operator was investigated in order to determine if deletion of the lambda sequence 3' from O_R1 would further decrease the affinity of repressor for O_R1 (Fig. 2c). The value of ΔG_1 determined for this operator was 1.4 kcal/mol more positive than that measured for the wildtype operator (Fig. 1; Table II). Taken together, the results of studies of the deletion mutants suggest that the lambda DNA-sequence on both sides of O_R1 contribute equally to the affinity of the site for repressor with proximity to a fragment end further altering (decreasing) binding affinity. The contribution of -1.4 of the -13.7 kcal/mol intrinsic binding energy by sequence outside O_R1 represents a significant effect on specific binding (i.e. an order of magnitude effect on binding affinity) and a large proportion of the free energy difference between specific and non-specific binding.

DISCUSSION

The thermodynamic validity of the individual-site binding curves determined by the footprint titration method under these conditions is well established (12,22). The application of individual-site binding methods (21) provides a rigorous theory for the accurate resolution of the intrinsic binding free energies for multi-site operators. The high quality of the binding data is evident in its excellent agreement with the Langmuir binding equation over the entire range of the titration. The precision of the data results in tight confidence limits to the resolved free energies (Table II). These confidence limits easily differentiate the 3 to 11 fold changes in equilibrium binding constants which were observed.

That the variation in binding free energies we observed is not due simply to the length of the restriction fragments is shown by two sets of experiments. First, repressor binds with equal affinity to the wildtype-operator containing restriction fragments of 1,102 bp and 648 bp (see Fig. 3a; manuscript in preparation). The equivalence of repressor binding to 1,102 bp and 648 bp fragments was also observed for several reduced-valency mutant operators (in preparation). Second, previous studies have shown that a series of restriction fragments isolated from pORI (Fig. 3b) of 155 bp, 412 bp, and 570 bp all bind repressor with equal affinity (12). Thus, analysis of a number of different operators show that binding affinity is independent of DNA-fragment length for the range of lengths which have been investigated in this study.

Since the studies presented in this paper are equilibrium binding assays, kinetic effects resulting from such processes as one-dimensional diffusion of repressor along the DNA would be manifest only if there were differential changes in the association and dissociation rate constants. Such differential changes as a function of DNA-fragment length have been reported for a number of DNA-binding proteins, including the repressor protein *cro* (24). Since *cro* and *cI*-repressor bind to the same set of sites in the O_R and O_L operators, the affect of fragment length of *cro* binding is of particular interest.

In our earlier studies a small *increase* in repressor affinity for a 120 bp fragment (Fig. 3b) was observed relative to the 155 bp fragment (12). This result is consistent with the

similar *increase* in *cro* affinity for a 73 bp fragment relative to fragments of 100 to several thousand bp (24). That the affinity of *cro* for O_R is also constant for longer DNA-fragments is suggestive of similarities in the mechanisms by which these two proteins locate, and bind to, specific sites. These *increases* in binding affinity for both proteins to short restriction fragments are opposite in direction to the *decrease* in repressor affinity for the 155 bp fragment from $pOR1$ relative to the 648 bp fragments containing wildtype and O_{R2^-} operators. Thus, the affinity differences we measure are not due to the lengths of the restriction fragments.

Two classes of models offer possible explanations for the effect of flanking sequences on specific binding by repressor. In the first class, repressor interacts directly with the flanking sequences. This model would require that the operator DNA conformation deviate significantly from linear DNA, possibly being bent upon repressor binding. The effect of flanking DNA-sequence on the affinity of the *E. coli* CAP protein is believed to be the result of this mechanism (25).

There is some evidence that repressor binding induces bending in the operator DNA. A gel electrophoresis analysis of whether repressor can induce bends in DNA (26) has not been published. The co-crystal structure of the N-terminal domain of repressor with a 20 bp oligonucleotide containing O_L1 shows very moderate bending of the DNA (10). Co-crystallization of repressor with longer DNA fragments would be required to critically test whether additional contacts are made with the flanking DNA. It is also possible that the interaction of the *intact* repressor with an operator site may differ significantly from the interaction of the *N-terminal domain* alone. When evaluating the energetic consequences of an x-ray diffraction structure it should be kept in mind that small changes in bond distances and angles often have large energetic consequences. Additional evidence suggestive of interaction of repressor with basepairs outside the canonical 17 bp site comes from hydroxyl radical footprinting studies (4). In these studies some protection of the DNA was observed 11 to 12 bp from the center of symmetry of O_{R1} .

In the second class of models, flanking sequences affect the conformation of O_{R1} , but do not interact directly with repressor itself. While it is clear from x-ray diffraction studies that sequence-dependent local changes in B-DNA structure exist (27), it is not clear whether long range perturbations in linear DNA can be propagated to distant sites along the sequence. NMR analysis of a single basepair substitution within O_{R3} revealed no spectral changes beyond the basepairs adjacent to the substituted site (28). On the other hand, evidence has been presented for a long range effect of a $(GC)_8$ insert within a 1,100 bp restriction fragment (29).

The decrease in binding affinity due the proximity of O_{R1} to a fragment-end is likely due to disruption of the structural integrity of the binding complex. Disruption of the binding complex could be due to transient strand separation or 'fraying' of the DNA helix.

The effect of the DNA-context on the affinity of repressor for O_{R1} suggests that studies of non-native binding-sites must be interpreted cautiously. For example, analysis of protein-DNA complexes using synthetic oligonucleotides as the operator DNA, in binding studies, NMR spectroscopy, or X-ray structural studies, may not accurately reflect the complete protein-DNA interaction. The effect of the DNA-context is particularly significant in the analysis of cooperative protein-DNA interactions. Resolution of the *intrinsic* and *cooperative* free energies which describe binding to multi-site systems requires the simultaneous analysis of wildtype and reduced-valency mutant operators (20–23). However, this analysis makes the crucial *assumption* that the intrinsic interactions at the remaining sites are unaltered by the changes made to inactivate a binding-site. Although usually not explicitly stated,

this assumption is also made in virtually all published studies on protein-DNA cooperativity.

The results of this study suggest that, in this case, a reduced-valency mutant created by single basepair mutation can be used for the analysis of cooperative interactions. However, this result may not be general and must be adequately tested for every system under study, preferably by the analysis of the effects of different mutations on protein binding. Simultaneous numerical analysis of the wildtype operator and either the deletion or synthetic operators shown in Table II would have yielded inaccurate cooperative free energies. The magnitude of the inaccuracy is large relative to the ca. 3 kcal/mol of cooperative free energy which has been measured (Table I). Thus, the internal consistency of the free energies resolved from different combinations of reduced-valency operators and wildtype operators must be tested to insure the accurate determination of cooperative free-energies.

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