Calorimetric Analysis of λ cI Repressor Binding to DNA Operator Sites†

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ABSTRACT: Enthalpies and heat capacities were determined by isothermal titration calorimetry for bacteriophage λ cI repressor binding to DNA containing various combinations of the three operator sites O_R1, O_R2, and O_R3 (each comprising a consensus half-site and a specific nonconsensus half-site). Differential scanning calorimetry was employed to evaluate the effects of specific DNA binding on thermal melting of the N-terminal and C-terminal repressor domains. Principal findings of this study are as follows: (1) Binding of repressor to each of the DNA operators is dominated by a large negative enthalpy, in agreement with earlier van't Hoff analyses of quantitative footprint titration data [Koblan & Ackers (1992) *Biochemistry 31*, 57−65]. The calorimetric data also revealed negative heat capacities for cI binding that are of comparable magnitude with many other systems [Spolar & Record (1994) *Science 263*, 777−784]. However, this feature in combination with the large negative values of binding enthalpies leads to an enthalpic dominance throughout the physiological temperature range. The resulting thermodynamic profile is opposite to the entropically dominated binding observed for many systems, including λ *cro* repressor which binds to the same sites as cI and also employs a helix−turn−helix binding domain [Takeda *et al.* (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 8180−8184]. It is suggested that these thermodynamic differences may arise from interactions of the cI repressor's N-terminal "arm" with the DNA. (2) Repressor monomers do not bind significantly to DNA containing either a consensus half-site or a nonconsensus half-site. Binding affinity to the double-consensus operator is much weaker than to any of the natural full-site operators. The same was found with other combinations of half-sites. A mutant repressor (PT158) which is severely defective in dimerization [Burz *et al.* (1994) *Biochemistry 33*, 8399−8405] was also found to bind only full-site operators and showed dimeric stoichiometry. (3) The thermal melting unit for N-terminal domains in the absence of DNA was found to reach values of 6−8 (monomer units) at concentrations where high-order oligomers of wild-type protein are formed [Senear *et al.* (1993) *Biochemistry 32*, 6179−6189]. However, in the presence of DNA operator sites, the cooperative unit for thermal unfolding was reduced to precisely two monomers, indicating that the N-terminal domain binds strictly as a dimer. (4) Significant nonadditivity was observed for the repressor binding enthalpies and heat capacities determined with multiple combinations of full-site operators. This effect was observed with wild-type cI and also with GD147, a C-terminal mutant repressor which is devoid of cooperativity but has normal binding affinity to each site [Burz & Ackers (1994) *Biochemistry 33*, 8406−8416]. Comparison of the results from wild type and GD147 suggests that repressor binding to the specific O_R sites may induce structural changes at neighboring sites which affect their interactions with repressor dimers. Possible structural origins of the thermodynamic effects found in this study are considered.

Interactions of phage λ cI repressor with the three DNA sites of O_R (the right operator) control the genetic switch between lysogenic and lytic modes of growth [cf. Ptashne (1992) and references therein]. Repressor protein cI binds as a dimer to each of the three 17 base pair DNA sites shown in Figure 1 using a helix−turn−helix motif (Pabo & Lewis, 1982; Jordan & Pabo, 1988). The dimeric repressors bind cooperatively to the three adjacent sites (O_R1, O_R2, and O_R3) ensuring precise control of transcription for the *cro* and cI genes from the promoters P_R and P_{RM} (Johnson *et al.*, 1979; Ackers *et al.*, 1982). This system has been widely studied as a prototype for elucidating the coordinated roles of protein−DNA recognition and cooperative interactions. Determination of the energetic driving forces between cI protein and the specific DNA operator sites is critical to understanding the relevant physical mechanisms of gene control.

A series of studies in this laboratory have been aimed at the physical chemistry of these interactions and their consequences for biological function [e.g., Shea and Ackers (1985), Koblan and Ackers (1992) and references therein, Burz *et al.* (1994), Burz and Ackers (1994), and Bain and Ackers (1994) and references therein]. Binding and cooperativity constants have been analyzed as a function of temperature by quantitative DNase I footprint titration (Koblan & Ackers, 1992). Over the temperature range 5−37 °C, the intrinsic binding of cI dimers to each of the three operator sites was found to be enthalpically driven, in contrast to many other protein−DNA interactions [cf. Spolar and Record (1994)]. Similar anomalous characteristics were subsequently determined for the binding of N-terminal domains (residues 1−102) to O_R sites (Bain & Ackers, 1994).

Whereas the initial crystal structure of the (unbound) cI dimer amino-terminal domain was found to be fully symmetrical (Pabo & Lewis, 1982), the cocrystal structure of

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FIGURE 1: Right operator sequences of bacteriophage λ showing specific repressor binding sites O_R1, O_R2 and O_R3 and the spacer sequences between sites. Consensus half-site sequences are in blue. (Note: 5′ and 3′ refer to the lower strand.)

the complex showed that cI dimers bind O_L1 DNA asymmetrically (Jordan & Pabo, 1988; Clarke *et al.*, 1991; Beamer & Pabo, 1992) and that only the N-terminal arm of the monomer at the consensus half-site makes significant contact with DNA. These observations motivated us to investigate the contribution of each half-site to the overall binding.

In the present study we have used calorimetric methods to determine the enthalpies and heat capacities of repressor−DNA binding and to extend the thermodynamic characterization of cI−O_R interactions. We were particularly interested in evaluating (1) the apparently anomalous thermodynamic behavior observed previously for these interactions, (2) the sequence requirements for efficient repressor binding, (3) the possibility of "long-range" effects among multiple operator sites within the same DNA molecule, and (4) the effects of operator DNA binding on the sizes of thermal unfolding units for the two repressor domains (i.e., the N-terminal and C-terminal domains). To explore these issues, we have also made use of mutant repressors that are defective in either dimerization (PT158) or cooperativity (GD147) (Burz *et al.*, 1994; Burz & Ackers, 1994).

MATERIALS AND METHODS

Chemical Reagents. Electrophoresis-grade acrylamide, ammonium persulfate, bis(acrylamide), and TEMED were obtained from Bio-Rad. CsCl was purchased from International Biotechnologies, Inc. (IBI), and was of molecular biology grade. All other chemicals were reagent or analytical grade.

Preparation of Proteins. Wild-type cI protein was purified as previously described (Johnson *et al.*, 1980). Purity was greater than 95% as judged by electrophoresis on SDS gels. However, the activity based on stoichiometric binding experiments described by Sauer (1979) was only 56%. Mutant GD147 and PT158 repressors were prepared as described in Beckett *et al.* (1993); activity was estimated at 87% and 55%, respectively. Concentrations of all proteins were calculated from the extinction coefficient at 280 nm (1.18 cm^{-1}·mL/mg).

Preparation of Operator DNA. Plasmid $_pO_R1$ DNA was purified using the procedure of Birnboim and Doily (1979), followed by CsCl density centrifugation (Maniatis *et al.*, 1982). Fragments of 570 bp containing site O_R1 were excised from the plasmid with *Eco*RI and isolated on a 15% agarose gel.

Synthetic Oligonucleotides. Oligonucleotides containing the plus and minus single-strand sequences of the right operator were purchased from Oligos Etc., Inc. (NJ), and Integrated DNA Technology (Cedar Rapids, IA). The two strands were purified using gel electrophoresis and annealed to double-stranded DNA by heating an equimolar mixture in 10 mM Bistris buffer (pH 7.0, 200 mM KCl) to 95 °C in a dry bath for 5 min and transferring it to a 65−70 °C water bath for several hours. The double-stranded DNA was separated from single-stranded DNA and purified on 15% polyacrylamide gel. To reduce end effects, all oligos were prepared with two additional base pairs at each end. The following sequences were synthesized: O_R1, AAATAGTGGCGGTCTCCATTT; O_R2, AAGTTGTGCGTGCCACAATTT; O_R3, AAATAGGGAACGCCACTATTT; nonspecific (NS), TGGAACCCACCGAGTGAAAGT; consensus half-site (CS), TTTATCACCGCAA; nonconsensus O_R1 half-site (NCS), TCTACCTCTGGAA; double-consensus site (DCS), TTTATCACCGCCGGTGATAAA. For multiple-site DNA, the natural λ O_R spacers were included, as shown in Figure 1.

Buffer. All protein and DNA samples were prepared in 10 mM Bistris (pH 7.00 ± 0.02 at $T = 20$ °C), containing 200 mM KCl, 2.5 mM $MgCl_2$, and 1.0 mM $CaCl_2$ (standard buffer SB).

Isothermal Titration Calorimetry. Calorimetric measurements were carried out using an Omega titration calorimeter (Microcal Inc., Northampton, MA), equipped with a nanovoltmeter. Data were analyzed using Origin, a software package provided by Microcal (Wiseman *et al.*, 1989). Enthalpies of interaction were obtained using single injection experiments. Ten microliters of DNA solution (15−100 μM) was injected into the sample cell ($V = 1.33$ mL) containing a stoichiometric excess of cI (0.5−3 μM). The heat of reaction was obtained by integration of the peak obtained after each injection. Control experiments were carried out by injecting the same amount of DNA into the reaction cell containing only buffer. These values were subtracted from the total heat of reaction to yield the net binding heat. The enthalpy of binding was obtained by dividing the heat of reaction by the number of moles of DNA injected. Reported enthalpies are averages of four to six independent injections. Additionally, complete titration curves were performed for the association of cI with single-site operators. The heat released from the ith injection is given by

$$\Delta q(i) = q(i) + \frac{dV_i}{V_0}\left\{\frac{q(i) + q(i-1)}{2}\right\} - q(i-1) \quad (1)$$

where dV_i is the volume injected and

$$q(i) = \frac{nM_t\Delta HV_0}{2}\left[1 + \frac{X_t}{nM_t} + \frac{1}{nKM_t} - \sqrt{\left(1 + \frac{X_t}{nM_t} + \frac{1}{nKM_t}\right)^2 - \frac{4X_t}{nM_t}}\right] \quad (2)$$

In eq 2, n is the number of sites per repressor dimer, M_t the total protein concentration in the cell, X_t the total concentration of DNA in the cell after injection i, V_0 the cell volume, ΔH the enthalpy of reaction, and K the association equilibrium constant. The measured isotherms were fitted to eq 2

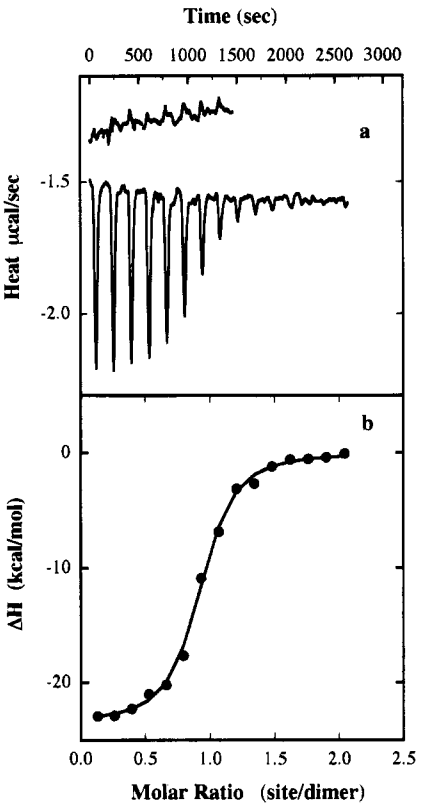

FIGURE 2: (a) Isothermal titration of wild-type cI repressor at 27 °C with (upper trace) the consensus half-site of O_R1 (13 bp; for sequences see Materials and Methods) and (bottom trace) the entire O_R1 site (21 bp). (b) Binding isotherm obtained by integrating individual peaks of the bottom curve in (a). The solid line represents the best fit as described in the text. Buffer conditions: 10 mM Bistris, 200 mM KCl, 2.5 mM $MgCl_2$, and 1.0 mM $CaCl_2$.

using a nonlinear least squares algorithm (Marquardt method), yielding estimates of the binding constant and reaction stoichiometry. To decrease the cross-correlation between fitted parameters, ΔH was obtained independently, as described above.

To evaluate the contributions of buffer ionization heats to the apparent heats of reaction, titration experiments were performed, at constant pH, in various buffers and the measured enthalpies plotted against reported heats of ionization (Christensen *et al.*, 1976). The slope of this plot yielded a value of 0.6 ± 0.3 mol of protons released by the buffer at pH 7.0 upon binding 1 mol of cI repressor dimers to site O_R1 (data not shown). This result agrees with previous studies by quantitative DNase I footprint titration which found the respective proton absorption values ($\Delta\bar{\nu}_H$) at pH 6.5 and 7.5 to be 0.4 ± 0.3 and 1.0 ± 0.2 (Senear & Ackers, 1990). As expected, the ionization heat of the Bistris buffer used throughout this study was found to be quite small (1.1 ± 0.4 kcal/mol). Figure 2 shows a typical titration curve and binding isotherm obtained from the reaction of wild-type protein with the 21 bp DNA fragment containing site O_R1 (Figure 1). Nonlinear least squares fitting yielded the stoichiometric values n (number of binding sites per repressor dimer) and the equilibrium constants, from which free energies were calculated. Binding enthalpies were obtained directly from single injection experiments and were held fixed in the fitting procedure.

Table 1: Thermodynamics of cI Repressor Binding to Individual Sites of O_R

DNA (bp)	$\Delta G°$ (kcal/mol)	$\Delta H°$ (kcal/mol)	$-T\Delta S°$ (kcal mol^{-1} deg^{-1})	n^b
	Calorimetric Results at 27 °C[a]			
O_R1 (570)	−11.9 ± 0.4	−28.4 ± 1.7	16.5 ± 2	1.17 ± 0.1
O_R1 (21)	−11.5 ± 0.4	−25.8 ± 1.3	14.0 ± 2	1.04 ± 0.1
O_R2 (21)	−9.8 ± 0.3	−15.2 ± 1.1	5.4 ± 1	0.89 ± 0.2
O_R3 (21)	−8.9 ± 0.4	−19.7 ± 1.2	10.8 ± 2	0.93 ± 0.1
nonspecific (21)	nd[d]	−1.7 ± 0.8	nd	nd
	van't Hoff Results at 30 °C[c]			
O_R1 (1102)	−12.4 ± 0.3	−23.3 ± 4.0	10.9 ± 4.3	
O_R2	−10.6 ± 0.2	−14.7 ± 1.4	4.0 ± 1.6	
O_R3	−9.9 ± 0.2	−22.7 ± 1.2	12.8 ± 1.4	

[a] Values are the mean and standard error (rms) of independent determinations. [b] Number of DNA binding sites per repressor dimer (corrected for protein activity). [c] Footprint titration data (Koblan & Ackers, 1992). [d] nd, not determined.

Table 2: GD147 Mutant Repressor Binding to Single Sites of O_R (Calorimetric Results at 20 °C)[a]

DNA	$\Delta G°$ (kcal/mol)	$\Delta H°_{GD147}$ (kcal/mol)	$-T\Delta S°$ (kcal mol^{-1} deg^{-1})	n^b
O_R1	−11.9 ± 0.3	−21.9 ± 1.4 (−22.5)[c]	10.0 ± 1.7	1.11 ± 0.13
O_R2	−10.1 ± 0.4	−13.4 ± 1.3 (−13.9)[c]	3.3 ± 1.7	0.94 ± 0.20
O_R3	−8.6 ± 0.3	−15.2 ± 1.3 (−17.3)[c]	6.6 ± 1.6	0.90 ± 0.10

[a] Values for GD147 are the mean and standard error (rms) of independent determinations. [b] Number of DNA binding sites per repressor dimer (corrected for protein activity). [c] Wild-type cI values estimated for 20 °C by interpolation of the linear plots of Figure 3.

Differential Scanning Calorimetry. Thermal denaturation curves were determined for the wild-type repressor, the DNA operator, and the protein−DNA complex by high-sensitivity differential scanning calorimetry using a Microcal MC2 instrument. We studied the effects of a single-site operator (a 30 bp sequence containing a single O_R1 site) on the thermal unfolding properties of wild-type cI repressor. Protein and DNA samples were dialyzed against the same buffer (SB). All experiments were performed at a protein concentration of 1 mg/mL. Samples were degassed under reduced pressure prior to scans. Analysis of the DSC data was carried out with the software Origin (Microcal Inc., Northampton, MA). The raw DSC curves were processed using standard procedures (Privalov & Potekhin, 1986). Each transition was analyzed independently. A cubic splines interpolation was used for baseline correction (Stearman *et al.*, 1988). Heat capacity curves were normalized to a concentration scale of monomer units.

RESULTS

Thermodynamics of Repressor Binding to Full-Site DNA Operators. Isothermal titration calorimetry provided direct determinations of the enthalpies for cI repressor binding to DNA containing various combinations of O_R sites and half-sites. Representative results from experiments on individual operator sites are given in Tables 1 and 2, along with calculated values of $\Delta G°$ and $T\Delta S°$. The calorimetrically determined values of n, corrected for protein activity, indicated that dimers are the active form of the repressor in binding to the operator sites, consistent with our previous studies using longer DNA fragments under similar conditions

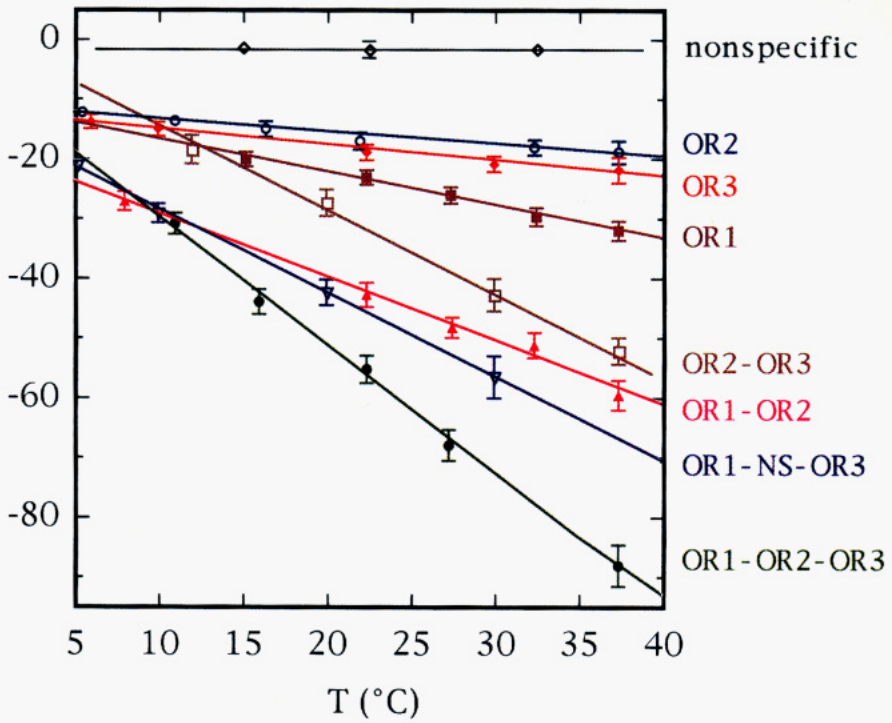

FIGURE 3: Enthalpies for wild-type repressor binding to oligonucleotides containing various combinations of O_R sites: black, nonspecific sequence; brown, O_R1; blue, O_R2; red, O_R3; pink, O_R1-O_R2; gold, O_R2-O_R3; purple, $O_R1-NS-O_R3$; green, $O_R1-O_R2-O_R3$. NS represents a nonspecific site (see Materials and Methods).

(Koblan & Ackers, 1992). The present calorimetrically determined binding free energies are slightly smaller than those obtained from the quantitative footprint titration data. Such differences might arise from slight "end effects" of the much shorter oligonucleotides employed in the calorimetric studies. However, the magnitude of these free energies is near the upper limit of the "measurement window" for calorimetry and is unlikely to be as accurate as those determined by footprint titration. By contrast, the reaction enthalpies, which are the parameters of primary interest in the present study, were determined with greatest accuracy by the calorimetric method.

Table 1 shows that the enthalpies of binding to sites O_R1 and O_R2 are in good agreement with those determined by van't Hoff analysis of quantitative footprinting data over the range 5−37 °C (Koblan & Ackers, 1992). With the indicated limits of statistical significance, the calorimetric value for O_R3 is only slightly smaller. Since titration calorimetry measures directly the enthalpy of reaction while van't Hoff analysis assumes a stoichiometric model, the agreement found between the two methods provides additional verification of the reaction stoichiometry used. The composite of results also shows that the repressor binding enthalpy depends only slightly on the length of the flanking DNA: the interactions of cI with either a 570 bp or a 21 bp oligonucleotide containing the same specific site, O_R1, are accompanied by similar enthalpy values. This finding is consistent with the value of −1.7 kcal/mol estimated for nonspecific binding (Table 1).

Listed in Table 2 are thermodynamic parameters obtained for binding the mutant GD147, containing a single-residue modificationin the C-terminal domain. This mutant has wild-type binding affinity for the three DNA sites but is devoid of cooperative interactions (Beckett *et al.*, 1993; Burz & Ackers, 1994). Data of the present study (Table 2) showed that GD147 repressor binds to each of the three individual operator sites with an enthalpy similar to that of wild type repressor.

Table 3: Heat Capacity Changes for the cI−DNA Interaction (kcal mol^{-1} deg^{-1})[a]

DNA	wild-type repressor	GD147 mutant repressor
O_R1	−0.55 ± 0.06	−0.48 ± 0.10
O_R2	−0.28 ± 0.06	−0.29 ± 0.08
O_R3	−0.27 ± 0.07	−0.29 ± 0.07
O_R1-O_R2	−1.45 ± 0.12	−1.26 ± 0.14
O_R1-O_R3	−1.50 ± 0.13	−1.00 ± 0.12
O_R2-O_R3	−1.60 ± 0.11	−0.80 ± 0.09
$O_R1-NS-O_R3$	−1.07 ± 0.10	−0.90 ± 0.10
$O_R1-O_R2-O_R3$	−1.96 ± 0.12	−1.67 ± 0.11

[a] Determined from slopes of ΔH vs temperature (Figure 3) over the range of 5−37 °C.

Combinations of Full-Site Operators. Temperature dependence of the binding enthalpies for wild-type cI to various combinations of O_R sites is illustrated in Figure 3. These enthalpies were found to remain negative at all temperatures over the range 5−37 °C. The data for each DNA fragment were fit well to a linear function, the slope of which yielded the standard heat capacity change for the respective combination of binding reactions. The resulting ΔC_p values are listed in Table 3. For comparison, the similarly determined heat capacity changes are also given for the mutant repressor GD147 over the same temperature range. At all temperatures studied the binding enthalpies of cI with multiple-site operators showed significantly stronger temperature dependence than with single operator sites (Figure 3, see Discussion).

Binding Experiments with Half-Sites. Isothermal titration calorimetry experiments illustrated in Figure 2 showed no binding to a DNA fragment containing the consensus sequence TATCACCGC. Similar results were obtained for tests of interaction with the nonconsensus half of O_R1. We

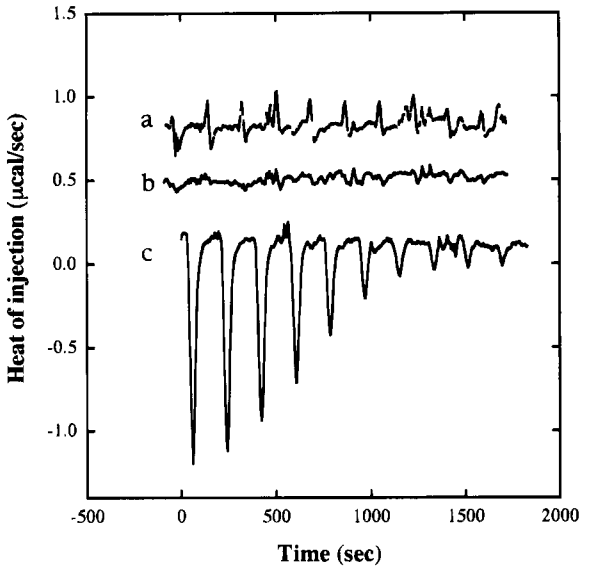

FIGURE 4: Titration of mutant repressor PT158 with (a) the consensus half-site (13 bp), (b) the nonconsensus half of site O_R1 (13 bp), and (c) the O_R1 site (21 bp) at 37 °C under standard conditions.

also investigated interactions of the mutant repressor PT158 with half-operator sites. This mutant is severely defective in dimerization and remains essentially monomeric up to 1 μM total protein under conditions similar to those used in this study (Burz & Ackers, 1994). However, footprint titration analysis showed that occupancy of both half-operator sites by PT158 occurs simultaneously; in the absence of a discernible dimer population, the DNA induces assembly of the dimers (Burz *et al.*, 1994). Here an 8 μM sample of DNA containing either a consensus half-site, a nonconsensus half-O_R1 site, or a full-site O_R1 was titrated with PT158 repressor (0.1−8 μM). The calorimetric data showed that PT158 also binds only to full-site operators, as illustrated in Figure 4. Analysis of these data showed a stoichiometry of $n = 1.1 \pm 0.2$ DNA binding sites per two repressors. Differential scanning calorimetry experiments on PT158 in the presence of operator O_R1 showed that the two binding domains unfold as a single cooperative unit (data not shown). These results suggest that when monomers bind to the operator sites, their interactions with DNA may be facilitating dimerization.

Combinations of Half-Sites. Binding of wild-type repressor by a symmetric double-consensus operator (see Materials and Methods) was assayed by isothermal titration calorimetry at 37 °C. It was found that the enthalpy decreased to −19.7 kcal/mol from the value of −30.0 kcal/mol found with the full O_R1 site. Further experiments showed that any other combination of half-sites decreased the binding enthalpy even more dramatically, suggesting that both the half-site sequences and their relative conformations may be critical for specific binding.

Effects of Operator Binding on Thermal Melting of Repressor. Thermal denaturation curves are shown in Figure 5 for the wild-type protein, a 30 bp duplex DNA containing site O_R1 and the repressor−DNA complex. The DNA melting curve showed a fairly sharp transition at 77 °C, in good agreement with the value of $T_d = 75.4$ °C calculated by the nearest neighbor method (Schildkraut & Shneior, 1965; Breslauer *et al.*, 1986). No other transition was observed at lower temperatures. The wild-type protein curve

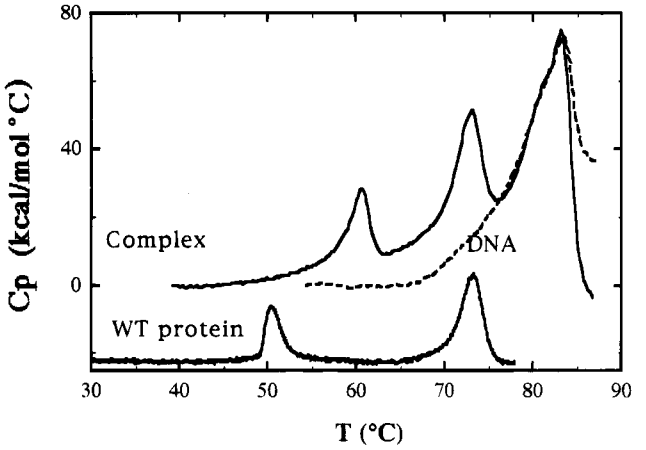

FIGURE 5: Experimental DSC scans: lower trace, wild-type repressor (38 μM); middle trace, a 30 bp DNA fragment containing the O_R1 sequence; upper trace, the cI−O_R1 complex.

Table 4: Parameters from DSC Scans on Wild-Type Repressor in the Absence and Presence of DNA[b]

domain	T_m (°C)	ΔH^a	$\Delta H_{vH}{}^a$	$\Delta H_{vH}/\Delta H$
		Absence of DNA		
N-terminal	50.7 ± 0.1	50.9 ± 0.4	332 ± 5	6.5 ± 0.2
C-terminal	73.3 ± 0.2	82.4 ± 3.8	348 ± 7	4.2 ± 0.3
		Presence of DNA		
N-terminal	59.4 ± 0.2	82.6 ± 5.2	168 ± 4	2.0 ± 0.2
C-terminal	73.0 ± 0.2	80.4 ± 3.0	299 ± 9	3.7 ± 0.4

[a] kcal/mol of monomers. [b] Protein concentration, 38 μM (monomer units); DNA ($O_R1-O_R2-O_R3$) concentration, 78 μM. Buffer: 10 mM Bistris, 200 mM KCl, 2.5 $MgCl_2$, and 1.0 mM $CaCl_2$ (pH 7.0).

(Figure 5) shows two apparent peaks, at 51 and 73 °C, corresponding to the N-terminal and the C-terminal domains, respectively. These results agree well with previous studies (Pabo *et al.*, 1979; Hecht *et al.*, 1985; Stearman *et al.*, 1988). Under present conditions, the unfolding transition of the N-terminal domain is totally reversible, and that of the C-terminal domain is approximately 88% reversible. The upper trace in Figure 5 represents thermal denaturation of the wild-type protein in the presence of a stoichiometric excess of specific DNA binding sites. It shows the transition temperature for N-terminal domains of the bound protein to be increased by almost 10 °C, relative to free protein. This substantial shift in T_m is expected for such tight binding (Snyder *et al.*, 1989). On the other hand, T_m of the C-terminal domains remained unchanged, consistent with previous findings that this domain does not interact directly with DNA (Pabo *et al.*, 1979).

The same picture is obtained when enthalpies of denaturation from the two calorimetric techniques are compared. In the presence of DNA, a large increase is found in the denaturation ΔH of amino-terminal domains while the C-terminal domain denaturation enthalpy remains unchanged (Table 4). It was of interest to compare the binding enthalpies derived from isothermal titration calorimetry (Figure 3, Table 3) with the DSC-derived values of denaturation enthalpies for N-terminal domains in the presence and absence of bound operator DNA. This difference enthalpy is obtained from areas under the respective DSC melting transitions (cf. Figure 5). For a three-site $O_R{}^+$ DNA fragment the enthalpy difference was found to be 31.7 kcal/mol. This value matches exactly the average intrinsic heat of binding to the individual operator sites (O_R1, O_R2, or O_R3)

extrapolated to 60 °C: $\langle \Delta H \rangle = [-25.8 - 0.55\ (60-27) - 15.5 - 0.28\ (33) - 19.7 - 0.27\ (33)]/3 = -32$ kcal/mol. This agreement suggests that only the intrinsic binding enthalpies add to the N-terminal denaturation enthalpy and that conformational changes of the DNA and protein−protein interactions mediated by the C-terminal domains do not contribute. Other contributions might include (a) the possibility that repressor dimers do not totally dissociate from DNA upon unfolding and (b) effects arising from the fact that although ΔC_p appears to be constant in the temperature range 5−37 °C, it is not known if it remains so to 60 °C.

Size of the Cooperative Unfolding Unit. The N-terminal denaturation data were analyzed to determine the size of the cooperative unfolding unit. It was found that these data were not in accord with a two-state thermal unfolding model which assumes independent melting of monomeric units (Privalov & Khechinashvili, 1974). In terms of calorimetric response functions the van't Hoff enthalpy ΔH_{vH} is determined by the heat capacity maximum ($C_{p,max}$), its temperature T_m, and the model-independent enthalpy ΔH, represented by the area under the transition peak:

$$\Delta H_{vH} = 4RT_m^2 C_{p,max}/\Delta H \qquad (3)$$

Results of this analysis are given in Table 4. In agreement with previous studies (Sauer *et al.*, 1986; Hecht *et al.*, 1986; Stearman *et al.*, 1988), we find the $\Delta H_{vH}/\Delta H$ ratio for the wild-type protein to be considerably greater than unity, indicating intermolecular cooperation among monomeric units in the unfolding process. It is well established that wild-type repressor dimers associate into higher order oligomeric forms above the micromolar range of subunit concentration. At a repressor concentration of 33 μM we found the van't Hoff to calorimetric enthalpy ratio to be 6.5 $\pm$ 0.2 for the melting transition of N-terminal domains (Table 4). When subunit concentration was increased to 60 μM, the ratio of van't Hoff to calorimetric enthalpies for the N-terminal transition increased to 8.1 $\pm$ 0.4, in accord with a predominance of octamers at this concentration. This is consistent with recent sedimentation studies which showed a dimer−octamer assembly mode with a tetramer as a possible intermediate (Senear *et al.*, 1993).

The denaturation transitions of wild-type repressor in the presence of operator DNA were also analyzed, after correction for the DNA melting curve. Results are summarized in Table 4. As noted earlier, the T_m of the amino-terminal domains was 60.03 °C, an increase of 9.3 °C over the transition temperature of free protein. The calorimetric enthalpy was also found to increase. However, the $\Delta H_{vH}/\Delta H$ ratio was found to be precisely 2. Thus in the presence of DNA, the cooperative unfolding unit of the N-terminal domain is strictly dimeric. This same ratio was found at a subunit concentration of 60 μM or when a longer DNA fragment was used (68 bp) containing sites O_R1, O_R2, and O_R3. The results of Table 4 also show that the T_m, the calorimetric enthalpy, and the $\Delta H_{vH}/\Delta H$ ratio of the C-terminal domains remain unchanged in the presence or absence of DNA. This is not unexpected since (1) the C-terminal domains do not bind DNA independently and (2) at the C-terminal melting temperature the N-terminal domain is unfolded and the protein is dissociated from the DNA.

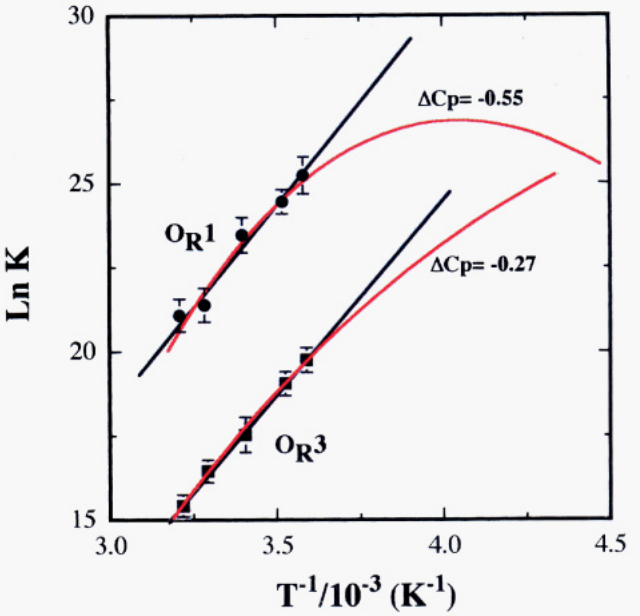

FIGURE 6: Calorimetric and DNase footprinting results transformed into van't Hoff representation for comparison. Data points are from quantitative footprint titration yielding best fit linear van't Hoff slopes (black lines). Red curves are calculated from the heat capacities determined in this study.

DISCUSSION

Correspondence between Results from Calorimetry and Footprint Titration. Previous footprint titration data showed a strong temperature dependence of binding free energies which, by van't Hoff analysis, yielded large negative enthalpies at each operator site over the same range as this study, 5−37 °C (Koblan & Ackers, 1992). Here we have used isothermal titration calorimetry to determine directly the enthalpies of cI−O_R interactions. The combination of calorimetric and footprint titration techniques provides highly complementary and mutually reinforcing information for complex systems such as this. Titration calorimetry employs enthalpy itself as the response function to measure interactions so that a direct determination is obtained for the net heat of all processes. These data yield values of ΔC_p by their temperature slopes. However, a partitioning of the overall heat effects into individual contributions within a complex system such as λ cI−O_R would be impossible without independent delineation of the behavior of site-specific and cooperative elements. A unique role of the footprint titration methodology lies in its ability to measure local-site binding isotherms and to resolve the stoichiometries and free energies among multiple, cooperatively interacting DNA sites. Analysis of the resulting free energies according to a stoichiometrically correct Gibbs−Helmholtz (or van't Hoff) relationship then provides valid enthalpies, as verified in Table 1. However, the evaluation of heat capacities for binding or cooperative interactions that may be reflected as only slight curvature in such van't Hoff plots is clearly out of the question.

These points are illustrated by Figure 6 which shows the reconciliation between the footprint titration results (Koblan & Ackers, 1992) and the isothermal titration calorimetry results of the present study (Figure 3, Table 3). Both of these data have been transformed into van't Hoff representation for comparison. Plotted points are from analysis of the footprint-derived values of K, the respective local-site binding

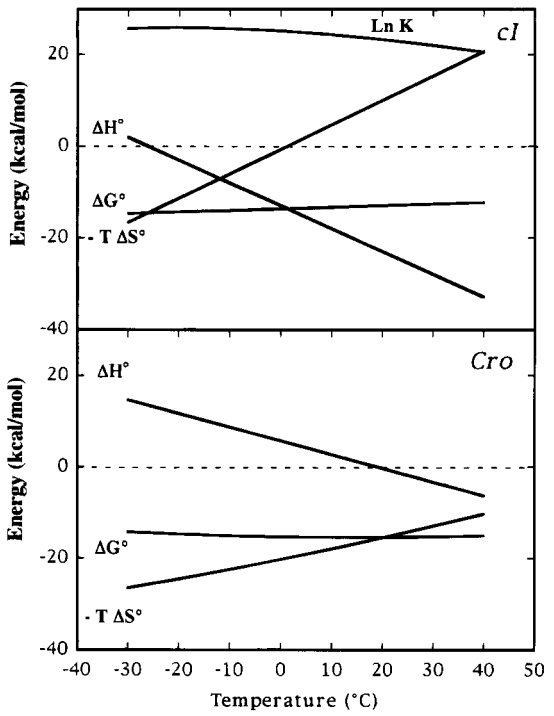

FIGURE 7: Thermodynamic profiles of cI repressor (top) and *cro* (bottom) binding to O_R1. The *cro* values are from Takeda *et al.* (1992).

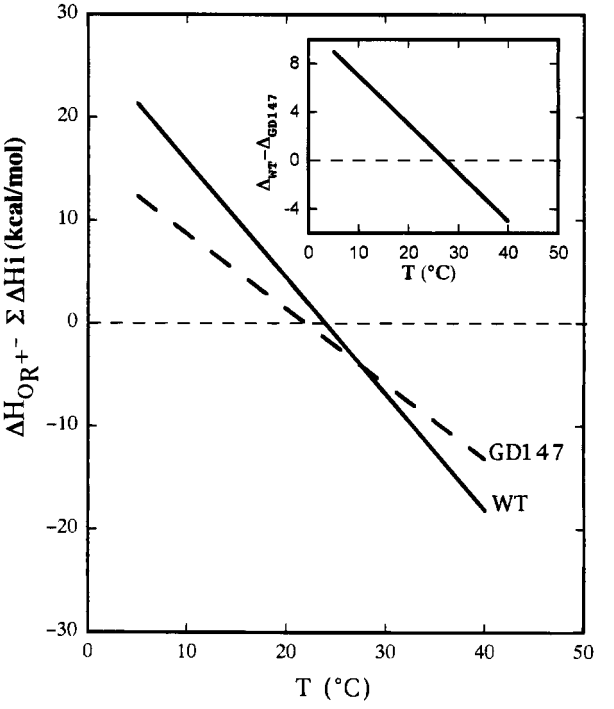

FIGURE 8: Nonadditivity of individual-site binding enthalpies (ΔH_{O_R} - $- \Sigma\Delta H_i$) for wild type (solid line) and GD147 (dashed line). O_R^+ is a 68 bp fragment containing all three specific sites in natural order, and $\Sigma\Delta H_i$ represents the sum of enthalpies determined with DNA fragments containing the individual sites. The insert shows the difference between nonadditivities of the wild type and GD147 repressors.

constants. These were determined using the three-site operator contained within an 1102 base pair DNA fragment. Linear van't Hoff fits are plotted in Figure 6 along with curves predicted from calorimetric results of Table 3 on the 21 base pair DNA fragments containing a single operator site (site O_R2 results were essentially identical to those of O_R3 and have been omitted from Figure 6 for clarity). It should be noted that the values of ΔC_p were obtained from calorimetric ΔH determinations at a sequence of temperatures within the same range as the footprint titrations (5−37 °C). The constant slopes of these plots for each operator (Figure 3) yielded accurate values of ΔC_p (Table 3). It can be seen (Figure 6) that the deviation from linearity predicted for the van't Hoff representation over the range 5−37 °C is so small that it falls well within errors of the footprint titration's linear van't Hoff function. Thus for each operator site the apparent binding enthalpies over the measurement range are in close agreement with those determined calorimetrically, as shown in Table 1. However, the footprint titration results are not capable of resolving (i.e., via their second derivatives) any of the heat capacities that have been found in the present study by calorimetry.

Thermodynamic Driving Forces for Repressor−DNA Interaction. Our previous delineation of the free energies of wild-type cI repressor binding to O_R1 (Kobland & Ackers, 1992) and the present calorimetric results on $\Delta H°$ and ΔC_p may be combined to illustrate the temperature-dependent changes in $\Delta G°$, $\Delta H°$, and $-T\Delta S°$ for the cI repressor−DNA system (Figure 7). In this treatment, we assumed ΔC_p to be independent of temperature, consistent with the data of Figure 3. (In general, ΔC_p would not be expected to remain constant over a very wide temperature range.) It is seen (Figure 7) that the enthalpic and entropic contributions to the free energy are strongly temperature dependent, but in a compensating manner so that $\Delta G°$ is nearly constant. This situation is expected for any process involving water as solvent and having a relatively large and negative ΔC_p. The values of T_H (the temperature at which $\Delta H° = 0$) and T_S (where $\Delta S° = 0$) are estimated as 247 and 273 K, respectively.

This profile contrasts sharply with those of a number of other protein−DNA interactions where entropic and enthalpic contributions to the free energy of molecular association have also been found to vary strongly with temperature (Jin *et al.*, 1993; Spolar *et al.*, 1994; Lundback *et al.*, 1994). In most cases, these contributions change sign in the physiological temperature range so that the association is entropically driven at low temperatures, but enthalpically driven at high temperatures. Such a thermodynamic signature is found with *cro* repressor (Takeda *et al.*, 1992), which binds to the same operator sites as cI, has similar affinities, and also utilizes a helix−turn−helix binding motif. Our results, however, indicate a different mechanism for the interactions of cI with DNA operator sites. Although the entropy changes are favorable at very low temperatures and strongly unfavorable at high temperatures, the binding process remains dominated by enthalpy at all temperatures in the physiological range. A recent calorimetric study (Ladbury *et al.*, 1994) found that the primary strong mode of binding of Trp repressor to operator DNA was also enthalpically driven throughout the physiological range (T_H = 6.6 °C), but an additional "secondary binding mode" having a large negative heat capacity was found to shift the apparent T_H to 15 °C.

Nonadditivity of Enthalpies and Heat Capacities. The difference between enthalpy of binding to the DNA fragment containing all three sites and the sum of binding enthalpies for the three respective single-site fragments is illustrated in Figure 8. Approximate additivity of the enthalpies is observed near 20 °C. However, at higher temperatures, the

enthalpy of interaction with each multisite operator is more negative than the sum of enthalpies for its constituent sites. For example, at 37 °C

$$\Delta H_{O_R^+} - \sum_{i=1}^{3} \Delta H_i = -13 \text{ kcal/mol}$$

On the other hand, at low temperatures, the nonadditive enthalpy is unfavorable. Similar effects are observed for the interactions of cI with the DNA fragments containing O_R1-O_R2 and O_R2-O_R3 compared to their constituent single-site values. Table 3 shows the differences in heat capacities for these systems. The ΔC_p differences are not caused by thermal instability of the oligonucleotides, as DSC scans show the oligonucleotides to be quite stable in the temperature range used for titration experiments of this study (e.g., Figure 5).

Possible origins of the observed nonadditivity in binding enthalpies and for the increased ΔC_p of the multisite fragments relative to their summed individual-site ΔC_p values include (1) favorable protein−protein contacts between repressor dimers bound at adjacent DNA sites and (2) ligation-induced structure changes of the DNA which alter the binding affinities at other sites. Protein−protein interactions involving C-terminal domains were shown in early studies to be responsible for cooperativity (Pabo *et al.*, 1979). These interactions can stabilize the complex by free energies of 2−3 kcal/mol (Ackers *et al.*, 1982; Brenowitz *et al.*, 1986), whereas the corresponding enthalpies of cooperative interaction are at least severalfold smaller (Koblan & Ackers, 1992). However, as noted earlier, the heat capacities of cooperative interaction among repressors bound at multiple sites are not determinable from temperature dependencies of the footprint titration data.

Several cI mutants with partial or total defects in DNA binding cooperativity have recently been isolated and characterized (Beckett *et al.*, 1993; Burz *et al.*, 1994; Burz & Ackers, 1994). Quantitative footprint titration studies showed that the GD147 repressor, bearing a Gly to Asp mutation in the C-terminal domain, binds with normal affinity to single operator sites but exhibits no cooperative free energy (Burz & Ackers, 1994). By their difference from wild-type cI values, the ΔH and ΔC_p values for GD147 binding to DNA containing combinations of the three operator sites may thus reflect the contributions of cooperative interactions to the total enthalpies and heat capacities. Indeed, the single-site binding enthalpies and heat capacities were found to be similar for GD147 and for wild-type cI (Tables 2 and 3). However, this was not the case for multiple-site DNA fragments (O_R1-O_R2, O_R2-O_R3, O_R1-O_R3, $O_R1-O_R2-O_R3$), which also showed significant differences in both ΔH and ΔC_p (Table 3). The difference between heat capacity changes for wild-type cI and GD147 is likely due to pairwise interactions between proteins bound at adjacent sites. This is consistent with the finding of essentially identical heat capacities for wild-type and GD147 repressors binding to the three-site fragment $O_R1-NS-O_R3$, containing a specific site at each and a nonspecific site in the middle (Table 3). Moreover, the measured heat capacity for $O_R1-NS-O_R3$ binding two repressor dimers was additive with respect to values obtained separately for sites O_R1 and O_R3. This additivity was found for both wild-type and GD147 repressors. These findings are consistent with the observed absence of interactions between dimers bound at sites 1 and 3, found in all footprint studies of wild-type cI binding to three-site operators containing a mutant template, O_R^{2-}, i.e., where a single base pair substitution in the middle site essentially eliminates repressor binding [cf. Senear and Ackers (1990)]. In those experiments the cooperative free energy ΔG_{13} for cI binding at the end sites, O_R1 and O_R3, was insignificant compared to the cooperative free energies of interaction between protein bound at adjacent sites (ΔG_{12} and ΔG_{23}). The composite of results from both footprint titration and calorimetry (i.e., Table 3) indicates that cooperativity always requires adjacent-site binding, but additional cooperativity arises upon three-site occupancy.

The data base of Table 3 indicates that protein−protein interactions cannot account for all of the observed nonadditivity in binding enthalpies and heat capacities. This is illustrated in Figure 8, which shows nonadditivity of binding enthalpy as a function of temperature for the GD147−O_R^+ and WT−O_R^+ complexes and the difference between them. This residual nonadditivity suggests that repressor binding to the specific O_R sites may induce structural changes at neighboring sites which affect their interactions with repressor dimers.

Effect of DNA Binding on Protein Stability and Self-Assembly. Our DSC results showed that, in the presence of operator DNA, the N-terminal transition is shifted to higher temperatures, while the C-terminal remains unchanged (Figure 5). This result adds to the numerous observations which indicate the N-terminal domain to be the structural element directly involved in binding. Also, investigation of the van't Hoff-to-calorimetric ratio of the N-terminal domain indicated that DNA dissociates wild-type repressor from high order oligomers to dimers. Recently, sedimentation equilibrium experiments of the wild-type protein in the presence of DNA suggested that octamers can bind between two and three oligonucleotides containing single operator sites without dissociating to dimers (Senear *et al.*, 1993). The present calorimetric data clearly indicate that, in the absence of DNA, the N-terminal domains form a cooperative unfolding unit consistent with an octameric aggregate but that, upon binding to DNA operator sites, this unit is reduced to a dimer. These findings may be consistent with those of Senear *et al.* (1993) if, for example, the observed octamer−oligonucleotide complexes are assembled through protein−protein contacts of the C-terminal domains.

Possible Roles of Noncovalent Interactions. The finding in this study that nonspecific binding is accompanied by only a small enthalpy is consistent with previous observations that nonspecific binding may be dominated by entropically driven electrostatic interactions (deHaseth *et al.*, 1977; Ha *et al.*, 1989; Manning, 1978; Record *et al.*, 1978). Also, our finding that nonspecific binding occurs with essentially zero heat capacity is consistent with calorimetric studies of DNA binding by *Escherichia coli* Trp repressor (Ladbury *et al.*, 1994) and *cro* repressor (Takeda *et al.*, 1992) where a nearly zero heat capacity was also found for nonspecific binding.

The negative heat capacity changes observed in this study for cI-specific binding are comparable to the values reported for other systems (Ha *et al.*, 1989; Jin *et al.*, 1993; Spolar & Record, 1994; Lundback *et al.*, 1994; Ladbury *et al.*, 1994; Takeda *et al.*, 1992). It is well-known that such negative values of ΔC_p may arise from hydrophobic effects. By

analogy with hydrocarbon transfer and protein folding, it has been suggested that "simple" protein−DNA associations may be dominated by hydrophobic interactions related to buried nonpolar surface but that conformational effects induced by the protein−DNA association may significantly alter this simplified picture (Ha *et al.*, 1989; Spolar *et al.*, 1994). That the protein−DNA interface is much more polar than the buried surface of proteins also suggests that hydrophobic interactions alone may not account for the large negative ΔC_p values observed. Recent studies have shown the difficulties in accounting for the observed ΔC_p on the basis of reduction in solvent-exposed nonpolar surface area upon complex formation (Ha *et al.*, 1989; Jin *et al.*, 1993; Ladbury *et al.*, 1994). Our results also suggest tht the hydrophobic effect is unlikely to be the dominant factor for cI−O_R interactions in the temperature range studied. Hydrogen bond formation, van der Waals interactions, and changes in protonation and conformation of the DNA and/or protein could all contribute to the observed negative enthalpy and negative entropy (Sturtevant, 1977; Ross & Subramanian, 1981). At pH 7.0, the 0.4 ± 0.3 mol of protons absorbed upon binding cI repressor to operator site O_R1 (Senear & Ackers, 1990) should contribute only a small part to the enthalpy of association. Since proton linkage to repressor binding is different for the three sites (Senear & Ackers, 1990), it is possible that proton−linked effects may account for a fraction of the observed enthalopy differences among the three operator sites.

Several hydrogen bonds may be formed upon binding cI protein to O_R sites, as seen from the cocrystal structure of the N-terminal domain bound to O_L1 (Jordan & Pabo, 1988). Moreover, the first six residues of λ cI comprise an N-terminal "arm" that makes multiple hydrogen bonds with the O_L1 site (Clarke *et al.*, 1991). The high-resolution (1.8 Å) structure showed that, in addition to Lys5 making a H-bond with the phosphate backbone, Lys4 participates in three hydrogen bonds, two to guanines and one to the oxygen of an amide side chain. Additionally, Lys3 forms H-bonds with two guanines in the major groove (G8 and G9) and makes direct contact with the extracyclic amino group of C8. The refined cocrystal structure showed that all contacts of the N-terminal arm are made with the consensus half-site bases of O_L1. Since these bases are also conserved on the other λ operator sites, similar interactions between the repressor arm and the right operator sites are likely.

These observations from the crystal structure could explain the large difference in cI binding enthalpy for sites O_R1 and O_R2. Their consensus half-site sequences differ at key positions, A3 and G9. A substitution at base pair 9 could not only eliminate contact with the arm at that position but also reduce contact of the arm with other bases. On the other hand, O_R1 and O_R3 consensus half-site sequences are identical, and the difference in binding enthalpies between sites O_R1 and O_R3 is smaller than the difference between sites O_R1 and O_R2. Therefore, H-bonds formed between the repressor arm and the consensus half-site of the operators could be responsible for a significant part of the negative enthalpy of association. Indeed, qualitative DNase I protection experiments at 4 and 37 °C showed that the binding of a truncated protein (amino acids 4−236) to O_R1 is less sensitive to temperature than is the binding of wild type (Eliason *et al.*, 1985), and deletion of the arm reduced to affinity by at least 8000-fold.

The enthalpy of wild-type cI binding to a symmetrical 21 bp DNA fragment containing the consensus sequence on both half-sites was found to be significantly smaller than the enthalpy for binding O_R1, suggesting that the sequence-dependent conformation of the operator site is critical for specific binding. Beamer and Pabo (1992) observed that an asymmetric structural variation in the nonconsensus half increases the helical twist required for an "induced-fit" mode of recognition.

The present calorimetric study demonstrates that sequence-dependent structural changes of the DNA operators play a significant role in the mechanisms of binding and cooperativity for λ cI repressor with O_R. Recent studies have suggested such a role (Benevides *et al.*, 1994; Bain & Ackers, 1994; Strahs & Brenowitz, 1994). The observed nonadditivities of enthalpies and heat capacities suggest that binding of cI to an individual site may induce conformational changes in the DNA that affect binding at other sites, with a consequent increase in heat capacity. This work also shows that the operator half-sites do not bind cI independently and that their relative position is critical for specific binding, suggesting again that DNA conformational changes play a significant role in both the mechanisms of intrinsic binding and cooperativity.

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