Use of Binding Enthalpy To Drive an Allosteric Transition†

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ABSTRACT: The *Escherichia coli* biotin repressor is an allosteric DNA binding protein and is activated by the small molecule bio-5′-AMP. Binding of this small molecule promotes transcription repression complex assembly between the repressor and the biotin operator of the biotin biosynthetic operon. The ability of the adenylate to activate the assembly process reflects its effect on biotin repressor dimerization. Thus concomitant with small molecule binding the free energy of repressor dimerization becomes more favorable by approximately −4 kcal/mol. The structural, dynamic, and energetic changes in the repressor monomer that accompany allosteric activation are not known. In this work the thermodynamics of binding of four allosteric activators to the repressor have been characterized by isothermal titration calorimetry. While binding of two of the effectors results in relatively modest activation of the dimerization process, binding of the other two small molecules, including the physiological effector, leads to large changes in repressor dimerization energetics. Results of the calorimetric measurements indicate that strong effector binding is accompanied by an enthalpically costly transition in the protein. This transition is "paid for" by the enthalpy that would have otherwise been realized from the formation of noncovalent bonds between the ligand and repressor monomer.

Many transcriptional regulatory proteins are subject to allosteric regulation. Classic examples include the lactose and purine repressors to which binding of a small ligand alters the affinity of the protein assembly for its target DNA sequence (*1*). In these systems the repressors are oligomers, and effector binding has no impact on the oligomeric state of the protein. Rather, the orientations/dynamics of the DNA binding domains respond to ligand binding at distal sites, thus altering the affinity of the protein for DNA. While in the lactose repressor system the affinity is decreased, the affinity of the purine repressor for DNA increases in response to effector binding. A second class of allosterically regulated transcription regulators includes those for which DNA binding affinity is modulated via altering the assembly properties of the protein. In these systems effector binding or posttranslational modification may be positively coupled to protein multimerization. Since the oligomeric species is often preferred in DNA binding, positive linkage of the allosteric signal to assembly increases the probability of occupancy of a transcription regulatory site of a gene. The diphtheria toxin repressor, DtxR, provides one example of this class of allosteric transcription regulators (*2*). In this system metal binding appears to enhance DNA binding by driving repressor dimerization. In contrast to the well-studied proteins such as LacR and PurR, which adhere to the "classical" allosteric mechanism, few of the transcriptional regulators in which the effector operates via its influence on protein assembly have been subjected to detailed physical−chemical analysis of the allosteric mechanism.

The *Escherichia coli* biotin regulatory system provides an excellent model for examining the allosteric activation mechanism in the latter class of transcription regulators. The central component of this system, BirA[1] or the biotin repressor, is a 35.3 kDa protein that possesses two biological functions (*3*−*5*). The two-step posttranslational addition of biotin to the biotin carboxyl carrier protein (BCCP) subunit of acetyl-CoA carboxylase catalyzed by BirA is essential for viability. In the first step the enzyme catalyzes synthesis of an activated biotin molecule, bio-5′-AMP, from the substrates *d*-biotin and ATP, and in the second the biotin moiety is transferred to a specific lysine residue of the carrier protein (*6*). BirA also functions to repress transcription initiation at the biotin biosynthetic operon (Figure 1). In this process two repressor monomers bind to a 40 base pair palindromic DNA sequence (*7*), bioO, thereby preventing transcription initiation at the two divergent overlapping promoters of the operon. Notably, it is not the aporepressor but rather the holo- or adenylate-bound species that is active in repression (*8*). Thus, bio-5′-AMP is both the active intermediate in enzyme-catalyzed biotin transfer and the positive allosteric effector of DNA binding.

The structure of the unliganded, or apo, repressor determined by X-ray crystallography revealed that it is a monomer and is organized into three domains (Figure 1) (*9*). The N-terminal domain adopts a winged helix−turn−helix motif and directly contacts the DNA in the repression complex. The central domain, which functions both in catalysis and

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[1] Abbreviations: BirA, *Escherichia coli* biotin repressor/biotin holoenzyme ligase; BCCP, biotin carboxyl carrier protein of acetyl-CoA carboxylase; bioO, biotin operator of the biotin biosynthetic operon; bio-5′-AMP, biotinoyl 5′-adenylate; btnOH-AMP, biotinol 5′-adenylate; btn-SA, 5′-*O*-[*N*-(biotinoyl)sulfamoyl]adenosine; Tris, tris-(hydroxymethyl)aminomethane.

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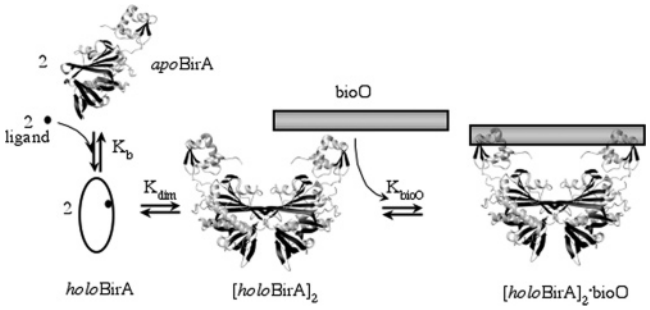

FIGURE 1: Allosteric activation of the biotin repressor by the small ligand bio-5′-AMP. Binding of the ligand is positively coupled to repressor dimerization. The resulting dimer binds site-specifically to the biotin operator sequence, bioO.

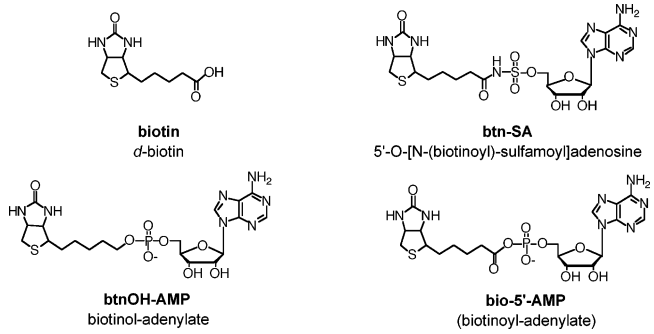

FIGURE 2: Chemical structures of the small molecule effectors biotin, btn-SA, and btnOH-AMP and the physiological corepressor bio-5′-AMP.

in DNA binding, is folded into a seven-stranded β-sheet and is also characterized by four partially unstructured surface loops. The C-terminal domain, to which no function has been definitively assigned, adopts a β-sandwich motif. As indicated above, the unliganded species is inactive in transcription repression.

The mechanism by which bio-5′-AMP binding allosterically activates the biotin repressor has been subjected to detailed investigation using equilibrium thermodynamic, kinetic, and structural approaches. First, as indicated above, two holorepressors bind to bioO (*10*). Second, binding of the adenylate to apoBirA is positively coupled to repressor dimerization (*11*). Results of sedimentation equilibrium measurements indicate that binding of the physiological corepressor, bio-5′-AMP, enhances dimerization of BirA by -4 to -5 kcal/mol (*12*). Moreover, the magnitude of this enhancement in dimerization energetics is equal to the enhancement in total assembly energetics of the repression complex from free holoBirA monomers and bioO (*12*). Furthermore, single amino acid substitutions at positions in three of the disordered surface loops of apoBirA result in loss of repression in vivo and defects in both dimerization and DNA binding in vitro (*13*). Finally, time-resolved DNase I footprinting measurements indicate that repressor dimerization precedes bioO binding (*14*). Thus, corepressor, bio-5′-AMP, binding regulates the repressor dimer supply, thereby dictating the probability of bioO occupancy and, therefore, levels of transcription initiation at the biotin biosynthetic operon.

The structure of the repressor bound to *d*-biotin, a weak allosteric activator of BirA dimerization and DNA binding, provides some insight into the structural origins of allosteric activation (*15*). In this structure the repressor is a dimer in which the protein−protein interface is formed by side-by-side alignment of the central domain β-sheets of each monomer. Interestingly, three of the four loops that are disordered in the apo structure appear ordered and at the monomer−monomer interface, a feature consistent with studies of single-site mutants summarized above. Additionally, the loop consisting of residues 110−128 is folded over and largely encloses the biotin molecule in the complex. These structural data are consistent with coupling of biotin binding to ordering of at least one of the loops that are disordered in the apo monomer. Combined results of solution and structural studies have prompted formulation of a model for allosteric activation of the repressor. Bio-5′-AMP binding drives a disorder-to-order transition in the surface loop(s) of BirA. This preorganization results in more favorable dimerization energetics, which, in turn, renders the overall assembly energetics of the transcription repression complex more favorable.

Linkage of repressor dimerization to DNA binding has been further investigated using corepressor analogues (*16*). The analogues were designed to function as unreactive mimics of bio-5′-AMP. Thus, the labile mixed anhydride connection between the *d*-biotin and AMP moieties was replaced with either a phosphate ester linkage (btnOH-AMP) or a sulfamoyl (btn-SA) linking group (Figure 2). The analogues bind to BirA with a 1:1 stoichiometry in the submicromolar concentration range and are inactive as substrates in the BirA-catalyzed biotin transfer reaction. Analogue function in allosteric activation of the repressor was probed by measuring the dimerization and bioO binding properties of BirA bound to each ligand (*16*). Results of sedimentation equilibrium measurements indicate that these effectors fall into two classes with respect to their abilities to promote repressor dimerization. Btn-SA, like *d*-biotin, is a weak allosteric activator of dimerization. In contrast, btnOH-AMP functions much like bio-5′-AMP and, upon binding to BirA, is a strong positive effector of the self-assembly process. Furthermore, results of DNase I footprinting measurements reveal a difference in the details of the allosteric mechanism for the strong and weak effectors. For strong effectors the magnitude of the enhancement of repressor dimerization associated with effector binding is equivalent to the magnitude of the enhancement in the overall free energy of repression complex assembly. By contrast, weak allosteric activators, biotin and btn-SA, exhibit no such equivalence. The accumulated data indicate that the critical species in the allosteric response is the liganded or activated repressor monomer. It is the dimerization properties of this species that dictate the occupancy of the biotin operator and, thus, the level of transcription initiation at the biotin biosynthetic operon. Therefore, further understanding of the allosteric activation mechanism requires information about the structural and dynamic changes that occur in the repressor monomer concomitant with effector binding.

Detailed thermodynamic analysis of the effector binding process provides one avenue for elucidating the mechanism of allosteric activation of the BirA monomer. In this work isothermal titration calorimetry has been used to dissect the energetics of BirA monomer binding to four allosteric activators. The molar Gibbs free energies, enthalpies, and entropies of binding have been determined. Results of these

measurements indicate that the effectors exhibit a range of Gibbs free energies of binding to BirA from −10 to −14 kcal/mol. Moreover, strong effectors exhibit binding enthalpies distinct from those observed for the weak effectors. While the two weak effectors bind with large favorable enthalpies, binding of each strong effector is characterized by a more modest enthalpic contribution to the total binding free energy. Linkage of protonation to the binding process is small for all ligands. Finally, binding of each effector to BirA is characterized by a small negative heat capacity change. However, no correlation is observed between the magnitude of the heat capacity change and the magnitude of the allosteric effect associated with a ligand. In the context of the model for allosteric activation, the contrasting enthalpic signatures of weak and strong effector binding indicate that activation of BirA for dimerization, and thus tight binding to bioO, requires an enthalpically costly transition in the protein. This transition is purchased with the enthalpy that should have been realized from the binding of the strong activating ligand to the repressor.

EXPERIMENTAL PROCEDURES

Chemicals and Biochemicals. All chemicals used in the preparation of buffers were obtained from commercial sources and were of at least reagent grade. The *d*-biotin was obtained from Sigma, and biotinoyl-5′-adenosine monophosphate (bio-5′-AMP) was synthesized and purified in this laboratory as described in Abbott and Beckett (*10*) using a modification of the procedure outlined in Lane et al. (*6*). The bio-5′-AMP analogues, 5′-*O*-[*N*-(biotinoyl)sulfamoyl]-adenosine (btn-SA) and biotinol adenylate (btnOH-AMP), were synthesized as described in Brown et al. (*16*) or were obtained from RNA-Tec (Leuven, Belgium). The biotin repressor (BirA) protein was overexpressed in and purified from *E. coli* BL21(λDE3)pHBA as described in Brown et al. (*16*). The purified protein was >95% pure as judged by SDS−PAGE and >92% active as determined using stoichiometric titrations with bio-5′-AMP monitored by fluorescence spectroscopy (*10*).

Isothermal Titration Calorimetry. All calorimetric binding measurements were performed using a VP-ITC microcalorimeter (1.44 mL cell volume) equipped with a thermovac sample degasser and a 250 μL syringe (MicroCal, Inc., Northampton, MA). Prior to performing each measurement protein stock was dialyzed extensively, unless otherwise indicated, against standard buffer [10 mM Tris-HCl (pH 7.50 ± 0.02 at 20.0 ± 0.1 °C), 200 mM KCl, 2.5 mM $MgCl_2$]. The dialyzed protein was filtered through a 0.22 μm PTFE syringe filter, and its concentration was determined spectrophotometrically using a molar extinction coefficient at 280 nm of 47510 M^{-1} cm^{-1} (*10*). The filtered dialysis buffer was used in preparation of all protein and ligand solutions, which were degassed for 10 min prior to use in titrations. In all experiments, the ligand was added to the protein in the sample cell. All heats of binding were corrected for the heat of ligand dilution by subtraction of the average heat associated with multiple injections of ligand performed after saturation of the protein. The binding data were analyzed using the Origin (7.0) software package.

Equilibrium Binding Titrations. (A) Direct Titrations. A direct titration method was utilized in calorimetric measurements of biotin and btn-SA binding to BirA. A 5 μM BirA solution was titrated with 29 10 μL volumes of either a 50 μM biotin or btn-SA solution in standard buffer at 20.0 ± 0.1 °C. The calorimetric signals were integrated, and nonlinear least-squares analysis of the resulting binding data using a single-site model was performed to determine the equilibrium association constant, K_A, the binding enthalpy, $\Delta H°$, and the stoichiometry of the interaction, *n*. The Gibbs free energy of binding, $\Delta G°$, and the entropic contribution to the binding free energy, $-T\Delta S°$, were calculated from the resolved parameters using the relationships:

$$\Delta G° = -RT \ln K_A \quad (1)$$

$$\Delta G° = \Delta H° - T\Delta S° \quad (2)$$

(B) Displacement Titrations. The high affinities of BirA for btnOH-AMP and bio-5′-AMP necessitated use of a displacement titration method for measuring these two binding interactions. This procedure was performed in two steps. First, a BirA solution was titrated to saturation with 29 10 μL volumes of a biotin stock solution. This titration was followed by a displacement titration with 29 10 μL volumes of a btnOH-AMP or bio-5′-AMP solution. The concentrations of components used for the btnOH-AMP titrations were 5 μM BirA, 50 μM biotin, and 50 μM btnOH-AMP. In the bio-5′-AMP titrations BirA was present in the cell at 2 μM, while the biotin and bio-5′-AMP titrant concentrations were each 20 μM.

The raw calorimetric signals obtained for each displacement titration were integrated and corrected for the heat of ligand dilution. The resulting binding isotherm was subjected to nonlinear least-squares analysis using a single-site model to obtain an apparent binding constant K_{app} and an apparent binding enthalpy ΔH_{app}. The binding constant K_A and enthalpy $\Delta H°_A$ for the higher affinity binding process are related to the apparent binding parameters by the relationships (*17*):

$$K_{app} = \frac{K_A}{1 + K_B[B]} \quad (3)$$

$$\Delta H_{app} = \Delta H°_A - \Delta H°_B \frac{K_B[B]}{1 + K_B[B]} \quad (4)$$

The parameters K_B and $\Delta H°_B$ refer to the equilibrium association constant and molar binding enthalpy for the ligand, in this case biotin, which binds to BirA with lower affinity. The parameters K_A and $\Delta H°_A$ correspond to the equilibrium association constant and binding enthalpy for the ligand that displaces biotin, either btnOH-AMP or bio-5′-AMP. The total biotin concentration in the cell is represented by [B].

(C) Titrations at Total Association. An alternative calorimetric titration method was used to obtain information about the heat capacity changes and linked protonation events associated with the four protein−ligand interactions. The method requires the injection of a total of 15 volumes of a concentrated ligand solution into the calorimeter cell containing the protein. An initial 2 μL injection is followed by five 5 μL injections, a single 150 μL injection, and finally eight 5 μL injections. The 2 μL injection is performed as a

precaution against any leakage of ligand solution that may have occurred during loading of the syringe into the calorimeter cell, and information obtained from this injection is discarded. Injections 2 through 6 were performed to obtain five measurements of the uncorrected binding enthalpy. The protein and ligand concentrations utilized are in the range of stoichiometric binding. Thus, in each of these five injections all ligand binds quantitatively to the protein. The injection of 150 μL is designed to fully saturate the protein so that the heat evolved in subsequent injections is due only to ligand dilution. The final eight injections were performed to obtain an average heat of ligand dilution. The average heat of these last eight injections was subtracted from each uncorrected measured heat of binding for injections 2 through 6 to obtain the corrected binding enthalpy, ΔH°_{bind}.

Using this method, one obtains five measurements of the binding enthalpy in one experiment. This strategy contrasts to the single-point saturation method used previously (*25*), which provides one measurement of the enthalpy and does not offer protection against leakage of the syringe during loading. The total association method was used to determine the binding enthalpy for formation of each effector complex as a function of temperature or the experimental buffer used.

Linkage of Protonation to Ligand Binding. The linkage of protonation (n_{H^+}) to ligand binding was investigated by measuring the dependence of the binding enthalpy on the ionization enthalpy of the experimental buffering agent. A BirA solution was titrated with a ligand in standard or MOPS buffer [10 mM MOPS (pH 7.50 ± 0.02 at 20.0 ± 0.1 °C), 200 mM KCl, 2.5 mM $MgCl_2$]. The number of protons released or taken up in the binding process was calculated from the measured heats of binding using the relationship (*18*):

$$\frac{\Delta H^\circ_{bind(A)} - \Delta H^\circ_{bind(B)}}{\Delta H^\circ_{ion(A)} - \Delta H^\circ_{ion(B)}} = n_{H^+} \quad (5)$$

where $\Delta H^\circ_{bind(A)}$ and $\Delta H^\circ_{ion(A)}$ are the measured binding enthalpy in buffer A (Tris-HCl) for formation of a specific BirA−ligand complex and the ionization enthalpy of buffer A [$\Delta H^\circ_{ion(Tris)} = +11.3$ kcal/mol] (*19*). Similarly, $\Delta H^\circ_{bind(B)}$ and $\Delta H^\circ_{ion(B)}$ are the measured binding enthalpy in buffer B (MOPS) and the ionization enthalpy of buffer B [$\Delta H^\circ_{ion(MOPS)} = +5.3$ kcal/mol].

Determination of Heat Capacity Changes of Ligand Binding. Heat capacity changes associated with formation of the protein−ligand complexes were determined by measuring the temperature dependence of the binding enthalpy for each process. With the exception of the bio-5′-AMP binding interaction, temperatures in the range of 5−30 °C were used for the measurements. The complications associated with ligand-induced dimerization necessitated limiting measurements of bio-5′-AMP binding to the range of 5−20 °C. The pH of each buffer was adjusted to 7.50 ± 0.02 at the working temperature. The heat capacity change upon complex formation is related to measured binding enthalpies and temperature by the equation:

$$\Delta C_p = \frac{\partial \Delta H^\circ}{\partial T} \quad (6)$$

Assuming that the heat capacity change associated with each binding process is constant in the temperature range employed, linear least-squares analysis of the dependence of the measured binding enthalpy on temperature yields the ΔC°_p for the binding process. Data analysis was performed using the Origin software package.

Table 1: Linkage of Ligand Binding to Biotin Repressor Dimerization

ligand	K_{dim} (M)[a]	ΔG°_{dim} (kcal/mol)[b]	$\Delta\Delta G^\circ_{dim}$ (kcal/mol)[c]
biotin	[7.0×10^{-3}]	[−2.9 (±0.4)]	−0.3 (±0.3)
btn-SA	1 (±0.1) × 10^{-3}	−4.0 (±0.1)	−1.4 (±0.2)
btnOH-AMP	3.8 (±0.8) × 10^{-5}	−5.9 (±0.1)	−3.3 (±0.2)
bio-5′-AMP	9 (±1) × 10^{-6}	−6.8 (±0.1)	−4.2 (±0.2)

[a] Equilibrium dimerization constants were determined for each BirA−ligand complex from a global analysis of six data sets obtained at two rotor speeds and three loading concentrations using sedimentation equilibrium. Experiments were performed in 10 mM Tris-HCl (pH 7.50 ± 0.02 at 20.0 ± 0.1 °C), 200 mM KCl, and 2.5 mM $MgCl_2$. Since biotin-induced dimerization is not detected under these conditions, the tabulated value is calculated from the value obtained in 50 mM KCl assuming that the salt dependence of dimerization for this complex is identical to that measured for the BirA−bio-5′-AMP complex (*12*). [b] The dimerization free energy was calculated by the relationship $\Delta G^\circ_{dim} = RT \ln K_{dim}$. [c] Differences in dimerization free energy relative to that measured for apoBirA, assuming that the enhancement of dimerization associated with ligand binding is independent of [KCl] (*12*).

RESULTS

Titrations of BirA with Biotin and Btn-SA. Titrations of BirA with the four ligands were performed to obtain the thermodynamic parameters for binding in the standard buffer conditions utilized in the laboratory. The relatively low affinities of the two weak allosteric activators, biotin and btn-SA, for BirA allowed use of direct calorimetric titrations. Moreover, the weak dimerization of each of these protein−ligand complexes eliminates (see Table 1) concern about any contribution of dimerization to the calorimetric signals obtained in titrations (*16*). Figure 3A shows a typical ITC profile for binding of btn-SA to BirA in standard buffer [10 mM Tris-HCl (pH 7.50 ± 0.02 at 20.0 ± 0.1 °C), 200 mM KCl, 2.5 mM $MgCl_2$]. The data were obtained from injecting 29 10 μL volumes of a concentrated btn-SA solution into the protein. Similar data were obtained in titrations of BirA with biotin. The binding isotherm generated from the data is also shown in Figure 3A along with the curve generated from the best-fit parameters resulting from nonlinear least-squares analysis of the data to a single-site model. The resolved thermodynamic parameters governing binding of biotin and btn-SA to BirA are provided in Table 2. The equilibrium dissociation constant determined for the BirA−biotin interaction is 4.2 (±0.3) × 10^{-8} M, a value in excellent agreement with that previously calculated from results of stopped-flow kinetic measurements of the kinetic parameters governing the interaction (*20*). The equilibrium dissociation constant for the repressor−btn-SA interaction indicates that this ligand binds to BirA with approximately the same affinity as does biotin. Additionally, while biotin binding and btn-SA binding are both characterized by large favorable enthalpies, the two binding processes are also opposed by sizable unfavorable entropic contributions to the binding free energy.

Displacement Titrations of BirA−Biotin with Bio-5′-AMP and BtnOH-AMP. Results of previous kinetic studies indicate

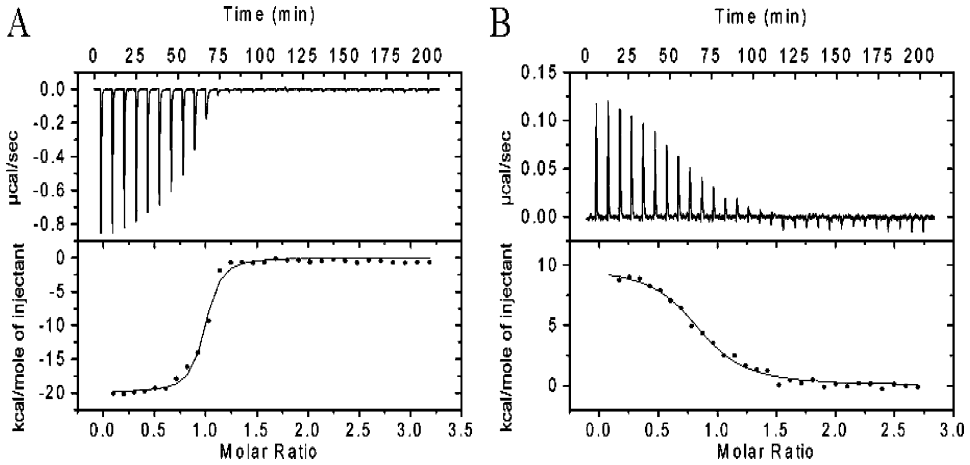

FIGURE 3: Calorimetric titrations of BirA. (A) Direct titration of BirA with btn-SA. (B) Displacement titration of BirA−biotin with btnOH-AMP. These experiments were performed at 20 °C in standard buffer [10 mM Tris-HCl (pH 7.50 ± 0.02 at 20.0 ± 0.1 °C), 200 mM KCl, 2.5 mM $MgCl_2$]. In (A) 29 10 μL volumes of a 50 μM btn-SA solution were injected into 5 μM BirA and in (B) 29 10 μL volumes of a 50 μM btnOH-AMP solution were injected into 5 μM BirA saturated with biotin.

Table 2: Thermodynamic Parameters Governing Effector Binding to the Biotin Repressor

ligand[a]	K_b (M)[b]	$\Delta G°$ (kcal/mol)[c]	$\Delta H°$ (kcal/mol)[b]	$-T\Delta S°$ (kcal/mol)[c]
biotin	$4.2 (\pm 0.3) \times 10^{-8}$	−9.89 (±0.04)	−19.2 (±0.1)	9.3 (±0.1)
btn-SA	$6 (\pm 1) \times 10^{-8}$	−9.7 (±0.2)	−19.9 (±0.3)	10.2 (±0.4)
btnOH-AMP	$1.5 (\pm 0.2) \times 10^{-9}$	−11.82 (±0.06)	−9.9 (±0.3)	−1.9 (±0.3)
bio-5′-AMP	$4 (\pm 2) \times 10^{-11}$	−13.9 (±0.4)	−12.3 (±0.9)	−1.6 (±0.9)

[a] All titrations were performed in 10 mM Tris-HCl (pH 7.50 ± 0.02 at 20.0 ± 0.1 °C), 200 mM KCl, and 2.5 mM $MgCl_2$ using a VP-ITC calorimeter (MicroCal, Inc., Northampton, MA). [b] Binding parameters K_b and $\Delta H°$ were obtained from the analysis of ITC data using a single-site binding model for a direct titration (biotin and btn-SA) or a displacement titration (btnOH-AMP and bio-5′-AMP) in the Origin software package. The parameters provided represent results obtained from a single titration with each ligand. In multiple titrations the resolved values of equilibrium constant and molar binding enthalpy are reproducible within 2-fold and 1 kcal/mol, respectively. [c] Binding free energies and entropies were calculated using the relationships $\Delta G° = RT \ln K_b$ and $\Delta G° = \Delta H° - T\Delta S°$.

bio-5′-AMP binds to BirA with an equilibrium dissociation constant of $5 (\pm 2) \times 10^{-11}$ M (*20*), a value beyond the utility of the direct ITC titrations (*21*−*23*). Moreover, preliminary measurements of the BirA−btnOH-AMP interaction indicated that this binding process is also not amenable to direct calorimetric titration. The displacement titration method has previously been applied to measure equilibrium dissociation constants in the picomolar range of concentration (*24*). In a displacement titration the protein is first saturated with a ligand characterized by a binding affinity sufficiently weak to measure by direct titration. In this case that ligand is biotin. The weaker binding ligand is then displaced by titration with a ligand that binds with a higher affinity. In the displacement titration the first ligand competes with the second ligand for the binding site, thereby yielding a reduced apparent affinity of the protein for the second ligand. Moreover, the measured heat of binding of the second ligand reflects both the intrinsic heat of binding of that ligand and the heat of displacing the first ligand. Therefore, to obtain a calorimetric signal characterized by an acceptable signal-to-noise ratio in a displacement titration, it is necessary that the enthalpic signatures for binding of the two ligands be distinct. For a detailed analysis of ligand competition protocol by displacement ITC, see Sigurskjold (*17*).

Results of a typical displacement titration of BirA−biotin with btnOH-AMP are shown in Figure 3B. The raw data represent 29 10 μL injections of a concentrated btnOH-AMP stock into the calorimeter cell containing BirA saturated with biotin in standard buffer at 20 °C. The calorimetric signal initially appears to indicate the occurrence of an endothermic process with each injection, up to the point at which the ligand/protein ratio is greater than 1. In subsequent injections, the signal becomes inverted, indicating the occurrence of exothermic events. Recall that the protein is initially saturated with biotin and binding of each btnOH-AMP molecule to BirA is coupled to displacement of a biotin molecule. As indicated above, biotin binding to BirA occurs with a large, favorable enthalpy of approximately −20 kcal/mol. Therefore, the endothermic peaks associated with the first several injections in the displacement of biotin by btnOH-AMP indicate that the ester binds with less favorable enthalpy than does biotin. In subsequent injections, as more binding sites become occupied by btnOH-AMP, fewer biotin molecules are displaced. Eventually the contribution of binding and displacement enthalpies to the observed calorimetric signal decreases, while the contribution from dilution of the ligand becomes apparent. The raw data were utilized to generate the isotherm also shown in Figure 3B. Nonlinear least-squares analysis of the data using a single-site model yielded the apparent binding enthalpy and equilibrium constant governing the reaction. The binding enthalpy of the high-affinity ligand in the displacement titration can be related to the apparent binding enthalpy using eq 4 (see Materials and Methods). The resolved parameters governing binding of btnOH-AMP obtained from the analysis are shown in Table 2. The equilibrium dissociation constant obtained for the ester analogue indicates that it binds to BirA approximately 4-fold more tightly than does biotin.

Titrations of BirA with bio-5′-AMP were also performed using the displacement technique. However, of the four

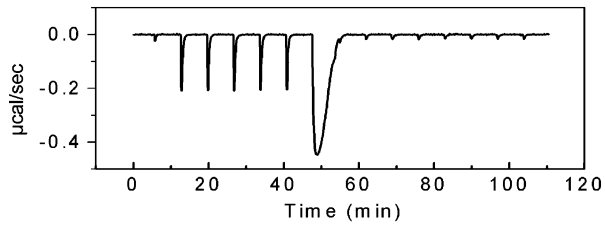

FIGURE 4: Titration of BirA with biotin under conditions of total association at partial saturation. The experiment was performed in standard buffer at 20 °C. A 2 μM BirA solution was titrated with a 20 μM biotin stock. The first 2 μL injection was followed by five 5 μL injections, one 150 μL injection, and, finally, eight 5 μL injections. The average integrated heat of the last eight injections was subtracted from the integrated heat of injections 2 through 6 to obtain corrected binding enthalpies.

ligands examined in this work, bio-5′-AMP binding to BirA has been shown to induce the tightest repressor dimerization. The equilibrium dissociation constant for the physiological holorepressor dimerization in standard buffer at 20 °C is approximately 10 μM (*11*, *12*, *16*). Additionally, measurements of the dependence of holorepressor dimerization on temperature reveal that it is an endothermic process (Brown and Beckett, unpublished data). It is therefore necessary to consider the potential contribution of the dimerization process to the calorimetric signal obtained in titrations with bio-5′-AMP. Consequently, a repressor concentration of 2 μM was employed for all bio-5′-AMP titrations. This concentration represents a compromise between minimizing the contribution of dimerization while optimizing the signal-to-noise ratio. However, in standard buffer at 20 °C approximately 23% of the 2 μM BirA−bio-5′-AMP complex forms a dimer. Thus, enthalpy values reported for bio-5′-AMP binding measured at 20 °C must be considered upper limits.

The displacement titration data obtained with bio-5′-AMP were analyzed in a manner identical to that obtained with btnOH-AMP, and the parameters governing its interaction with BirA are provided in Table 2. The equilibrium dissociation constant obtained for the process of 4 ($\pm$2) $\times$ 10^{-11} M agrees well with that calculated from kinetic measurements of the interaction (*20*). Moreover, binding of each ligand, btnOH-AMP and bio-5′-AMP, to BirA is accompanied by a favorable enthalpic contribution. However, consistent with the endothermic peaks observed for the initial injections of btnOH-AMP and bio-5′-AMP in displacement of biotin from BirA, the magnitude of the binding enthalpy for each of these ligands is approximately half of that observed for biotin and btn-SA. Furthermore, each of these strong activators binds to the repressor with a small, but favorable, entropic contribution to the Gibbs free energy.

Titrations at Total Association. To obtain information about heat capacity changes associated with the four binding interactions as well as the linkage of protonation to each process, an alternative method was utilized to measure the binding enthalpy. This method is a modification of the single-point saturation method previously utilized (*25*). Figure 4 shows the raw data obtained from a titration of BirA (2 μM) with a concentrated biotin stock solution (20 μM) in standard buffer at 20 °C. The first peak represents the heat evolved upon injection of a 2 μL volume of the syringe solution into the calorimeter cell containing the protein. The purpose of this injection is to expel any volume of air that may have been generated by vibration while inserting the syringe into the calorimeter cell. The actual volume of ligand delivered in this injection is at most 2 μL and is dependent upon the extent of the leakage that may have occurred from the syringe. Therefore, any heat measured for this injection is unreliable and is not used in data analysis. The next five peaks and the final eight peaks represent the heat evolved upon the addition of a 5 μL volume of the ligand solution. The large peak represents the heat evolved upon injection of 150 μL of ligand solution into the calorimeter cell. Injections 2 through 6 were performed to obtain several measurements of the heat of binding. On the basis of the equilibrium dissociation constant for biotin binding of 4.2 ($\pm$0.3) $\times$ 10^{-8} M, in each of these injections all of the biotin binds to BirA. This stoichiometric binding condition has been termed *total association at partial saturation* (*26*). The observed signal associated with each injection, however, reflects the combined heat of binding and heat of ligand dilution. The large volume injection was performed to add sufficient biotin to fully saturate the protein. The remaining eight peaks represent the heat evolved upon the addition of biotin after saturation of the protein and, therefore, simply provide a measure of the heat of dilution of the ligand. The integrated heat determined for each of the injections 2 through 6 is corrected for ligand dilution by subtracting the average of the integrated heat associated with the final eight injections. In this manner one obtains five measurements of the binding enthalpy from one titration experiment.

This method is desirable for measuring binding enthalpies for several reasons. First, in contrast to the single-point saturation method previously employed, the standard precaution is taken to eliminate uncertainty in the volume of the first injection due to possible leakage during loading of the syringe into the calorimeter. Second, the single-point method and a full titration provide only one estimate of the binding enthalpy, while, using the modified method, five estimates can be obtained in a single experiment. Thus information about the precision of the measurement can be obtained. A final advantage is that, in contrast to the full titration in which two parameters, the equilibrium constant and the enthalpy of binding, are resolved in data analysis, only the enthalpy value is obtained from a titration performed by this method. As a result a higher accuracy is associated with the resolved enthalpy values. This method was employed to measure all enthalpies used in determination of heat capacity changes and linked protonation events for binding of each to the biotin repressor.

Linkage of Protonation to Binding. To estimate the linkage of protonation to binding of each ligand to BirA, the dependence of the binding enthalpy on the buffer ionization enthalpy was measured. The buffer utilized in the experiments contained 200 mM KCl and 2.5 mM $MgCl_2$, with either 10 mM Tris or MOPS as the buffering agent. Data were analyzed using eq 5 to estimate the number of protons taken up or released upon binding of each ligand to BirA (Table 3).

Results of the measurements indicate a range of dependencies of binding enthalpies on the buffer ionization enthalpy. The BirA−btnOH-AMP interaction is characterized by an n_{H^+} value of zero. Binding of the weak effectors, biotin and btn-SA, is coupled to the uptake of protons, and by contrast, bio-5′-AMP binding is linked to proton release. Small linked

Table 3: Heat Capacity Changes and Linked Protonation in Ligand Binding to the Biotin Repressor

ligand	ΔC°_p [cal/(mol K)][a]	n_{H^+}[b]
biotin	−206 (±12)	−0.14 (±0.04)
btn-SA	−395 (±16)	−0.44 (±0.08)
btnOH-AMP	−363 (±14)	0.04 (±0.05)
bio-5′-AMP	−183 (±22)	0.34 (±0.05)

[a] The heat capacity changes were determined from the slope of a linear least-squares analysis of the temperature dependence of the enthalpy for binding of each ligand to BirA in 10 mM Tris-HCl, 200 mM KCl, and 2.5 mM $MgCl_2$. The pH of the buffer was adjusted to 7.50 ± 0.02 at the experimental temperature. Ten values of the binding enthalpy measured at each temperature were used in each linear regression to obtain ΔC°_p. [b] The linkage of protonation to binding was determined from the slope obtained from linear least-squares analysis of the dependence of the binding enthalpy on the buffer ionization enthalpy. Ten measurements of the binding enthalpy obtained in each buffer were used in the linear regression to obtain n_{H^+}.

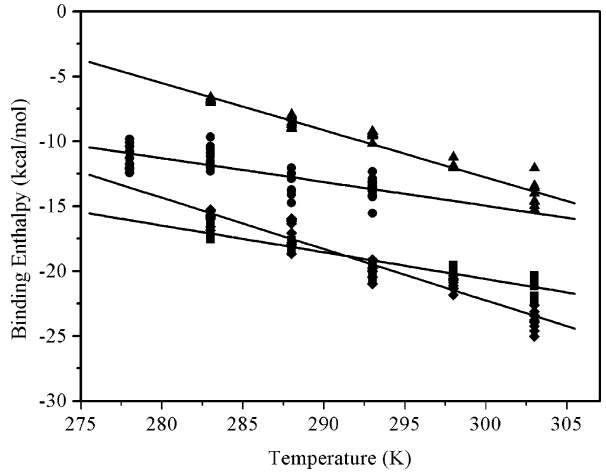

FIGURE 5: Heat capacity changes for binding of the four ligands to BirA. A plot of the temperature dependence of the binding enthalpy for (■) biotin, (◆) btn-SA, (▲) btnOH-AMP, and (●) bio-5′-AMP. Experiments were performed as described in Figure 4 at the indicated temperatures.

protonation effects are observed for all four binding processes.

Heat Capacity Changes. The heat capacity change associated with each ligand binding process was obtained from measurement of the binding enthalpy as a function of experimental temperature. Each complex of BirA bound to biotin, btnOH-AMP, or btn-SA is characterized by weak dimerization. Thus, the self-association reaction made no contribution to the heats measured by ITC. By contrast, as indicated above, the contribution of dimerization to the measurements of bio-5′-AMP binding enthalpy was of potential concern. However, measurements of the temperature dependence of the equilibrium dimerization constant for this complex indicate that the process becomes weaker with decreasing temperature (Brown and Beckett, unpublished observations). Thus, concern about any contribution from the dimerization enthalpy to measured binding heats was eliminated by limiting the binding measurements to 5−20 °C. Linear least-squares analysis of the dependence of the measured binding enthalpies on experimental temperature (Figure 5) yielded the heat capacities displayed in Table 3. The results indicate that all four binding interactions are characterized by negative heat capacity changes. The magnitudes of the changes are small, and no correlation exists between the magnitude of the heat capacity change and the magnitude of the allosteric effect induced by a ligand.

DISCUSSION

Solution and structural analyses of the biotin regulatory system indicate an allosteric activation model in which binding of bio-5′-AMP to BirA drives self-assembly of the repressor, thereby promoting transcription repression by increasing the occupancy of the biotin operator site. Recent investigations of the allosteric response associated with binding of *d*-biotin, bio-5′-AMP, and the two adenylate analogues (btn-SA and btnOH-AMP) to the repressor reveal that the ligands fall into two classes: weak and strong effectors. Strong effector, btnOH-AMP or bio-5′-AMP, binding results in substantially enhanced repressor dimerization energetics, and this enhancement is quantitatively reflected in the measured total assembly energetics for the transcription repression complex. In contrast, for *d*-biotin and btn-SA, which weakly enhance dimerization, uncoupling of dimerization and DNA binding processes is observed. In the context of the experimental data thus far accumulated, this uncoupling indicates that, in addition to the dimerization surface, the allosteric response to effector binding by BirA involves other regions of the protein structure. Furthermore, elucidating the structural and dynamic changes that accompany effector binding to the repressor monomer is central to understanding the allosteric activation process.

The measurements presented in this work were designed to determine the thermodynamic profiles associated with binding of the four effectors to the repressor monomer and to examine the relationship, if any, of these profiles to allosteric activation. All four ligands bind tightly to BirA with equilibrium dissociation constants in the nanomolar to picomolar concentration range. The Gibbs free energy of *d*-biotin binding of −9.89 (±0.04) kcal/mol agrees well with values previously determined using kinetic methods (*20*) and titration calorimetry (*25*). Btn-SA also binds BirA with an equilibrium dissociation constant in the nanomolar range of concentration. The tight binding of bio-5′-AMP and btnOH-AMP to BirA necessitated use of the displacement titration method in measurements of binding of these two ligands. The Gibbs free energy for binding of bio-5′-AMP obtained with this method is −13.9 ± 0.4 kcal/mol, a value in close agreement with that calculated previously from results of kinetic measurements (*20*). The ester analogue binds to BirA with a Gibbs free energy that is intermediate between that measured for *d*-biotin and bio-5′-AMP. These latter results demonstrate the utility of the displacement titration method for measuring tight binding interactions.

The binding free energies for association of the four ligands with BirA can be considered in relation to the known structure of the biotin-bound repressor. In this structure the biotin moiety is located in a pocket on one face of the β-sheet of the central domain and is largely solvent-excluded. The loop composed of residues 112−128, of which residues 116−124 are disordered in the aporepressor structure, forms a lid over the ligand. The conformation of this loop is stabilized by the formation of several hydrogen-bonding interactions between the main chain and side chain residues of the loop and the ureido group of biotin and by a backbone amide hydrogen bond of arginine 118 with the biotin carboxylate.

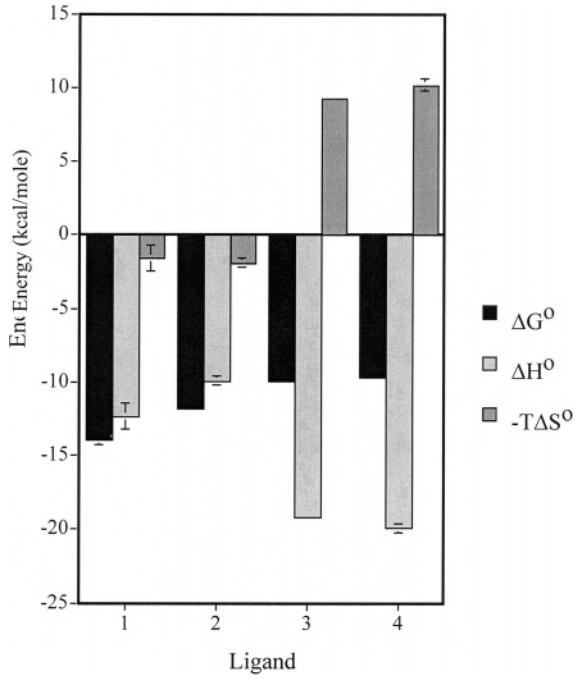

FIGURE 6: Distinct thermodynamic profiles for binding of the strong (1) bio-5′-AMP and (2) btnOH-AMP and weak (3) btn-SA and (4) biotin effector ligands to the biotin repressor.

Three glycine side chains interact with the thiophene ring, and a tryptophan side chain stacks against the valerate chain of biotin. Similar bonding interactions between the biotin moiety and the protein are likely to exist in the complexes of BirA bound to each of the remaining three effectors. Therefore, the similar affinity for BirA exhibited by biotin and btn-SA, which is characterized by the sulfamoyladenosine appendage that provides more functional groups for potential hydrogen-bonding and π-stacking interactions, is puzzling. The other two adenylates, the btnOH-AMP and the bio-5′-AMP, bind with significantly higher affinity to BirA. While the Gibbs free energy for anhydride binding is -4.0 kcal/mol more favorable than that for btn-SA binding, the ester binds with a Gibbs free energy that is -2.0 kcal/mol more favorable. Perhaps the reduced affinity of btn-SA relative to btnOH-AMP and bio-5′-AMP reflects the absence of a charge at the linker region between the biotin and adenosine moieties. Alternatively, the conformation of the sulfamoyl derivative compromises interactions between the protein and the adenylate and/or biotin moiety. Binding of bio-5′-AMP to BirA is -2 kcal/mol more favorable than is binding of btnOH-AMP, which differs structurally from bio-5′-AMP only by the replacement of the valerate carbonyl group by a methylene. This result indicates that the carbonyl group at this position is significant for the binding process.

The results of isothermal titration calorimetry measurements allowed dissection of the Gibbs free energy of binding of each ligand to BirA into its enthalpic and entropic components. As indicated above, the four effectors fall into two classes with respect to their efficacy in promoting BirA dimerization. In addition to their distinct strengths in allosteric activation, the enthalpic and entropic components of the binding energy for the four BirA complexes also fall into two classes (Figure 6). While the weak allosteric effectors bind to BirA with large favorable enthalpies, the strong effectors bind with relatively modest enthalpy changes. Furthermore, the weak effectors bind to BirA with significant unfavorable entropy changes while entropy moderately enhances strong effector binding.

Similar enthalpic changes are associated with binding of the two weak effectors to BirA. If the same contacts are made between the protein and the biotin moiety of btn-SA as are made in the BirA−biotin complex, one might expect a larger enthalpic change for btn-SA binding due to the additional bonding potential associated with the sulfamoyladenosine moiety. The absence of this additional enthalpic gain for btn-SA binding may reflect the absence of a charged functional group that can interact with the repressor as observed in the BirA−biotin structure. The strong allosteric effectors contain the adenosine functionality as well but bind with approximately one-half of the enthalpic contribution that characterizes biotin or btn-SA binding. Again, in considering solely the bonding potential of the ligands, one might anticipate that addition of the adenosine group would result in a larger enthalpic contribution to binding. In comparison to the weak effectors, it appears that there is an enthalpic penalty associated with binding of strong effectors to BirA.

The unfavorable entropy changes for binding of biotin and btn-SA are consistent with the notion that loops in the protein become organized in the course of ligand binding. Organization of the loops concomitant with binding restricts the number of conformational and translational degrees of freedom that these loops can explore. This restriction is entropically unfavorable for the protein as well as the ligand. By contrast, the small favorable entropic contributions associated with binding of btnOH-AMP and bio-5′-AMP are inconsistent with binding processes dominated by the known folding of the protein loops. The coupling of other changes in the protein and/or solvent to binding of the two ligands presumably provides the entropic driving force for the reactions.

The contrasting thermodynamics of binding weak and strong effectors can be considered in the context of drug design strategies. In designing inhibitors of protein targets, it is common to try to maximize the favorable enthalpy of binding by optimizing complementarity between a target protein and a compound. Indeed, a correlation between the strength of interaction of compounds with HIV-1 protease and the magnitude of the favorable enthalpic contribution to the binding has been noted (*27*). The results described in this work suggest that maximizing enthalpy may not always provide the optimal affinity of a ligand for a target protein. The two tight-binding effectors, btnOH-AMP and bio-5′-AMP, are characterized by modest binding enthalpies when compared to the weaker binding biotin and btn-SA. Switching from low to high affinity in this system is accomplished with less favorable binding enthalpy and more favorable entropy. Moreover, in considering the functional consequences of binding, maximal allosteric activation is accomplished with ligands that bind with modest enthalpy.

Heat capacity changes associated with binding of the four ligands are small and negative. Moreover, the values determined from these measurements of biotin and bio-5′-AMP binding are in good agreement with those previously reported for those two ligands (*25*, *28*). In considering these heat capacity changes, no correlation exists between its magnitude and the efficacy of a ligand in activation of repressor dimerization. The magnitudes of heat capacity

changes in allosteric systems, because of their relationship to burial of hydrophobic and polar surface area, are frequently interpreted in terms of conformational changes accompanying ligand binding. However, the heat capacity changes obtained for the four ligands indicate that, while biotin or bio-5′-AMP binding is each characterized by relatively small values, btn-SA and btnOH-AMP binding are characterized by relatively large values. By contrast, the functional differences of the complexes with respect to dimerization suggest that the conformations of the repressor bound to biotin or btn-SA should be similar but distinct from those in which bio-5′-AMP or btnOH-AMP is bound.

To relate the thermodynamic parameters to allosteric activation, it is necessary to correlate structural changes in the repressor that occur upon binding of the effector to the energetics of self-association of the liganded monomer. In comparing the three-dimensional structures of the biotin-bound dimer and the apo monomer, differences are localized to three of the four flexible surface loops. However, biotin is a weak allosteric activator of dimerization, and the structure of the monomer bound to bio-5′-AMP is probably distinct from that of the *d*-biotin-bound monomer. First, the two complexes are functionally different with respect to dimerization. Second, results of partial proteolysis and hydroxyl radical probing of apoBirA, BirA−biotin, and BirA−bio-5′-AMP indicate that their monomeric states in these complexes are structurally distinct. The BirA surface loop composed of residues 212−236 that remain disordered in the biotin-bound structure is significantly more resistant to subtilisin cleavage in the adenylate-bound protein than it is in the apo- or biotin-bound form (*15*, *20*). Finally, results of studies of a truncated form of BirA from which the N-terminal domain is deleted are consistent with a ligand-dependent interdomain interaction between the DNA binding and central domains (*29*). Thus, conformational differences between the adenylate- and biotin-bound monomers may be localized, in part, to an interdomain interface between the N-terminal and central domains of the protein.

The distinct thermodynamic signatures for binding of the strong and weak activators reinforce the idea that the adenylate-bound repressor is structurally distinct from the biotin-bound repressor. Moreover, data indicate that the conformational transition associated with activation of the monomer for dimerization is enthalpically costly. Binding of the two strong allosteric activators is characterized by a moderate enthalpy relative to binding of the weak activators. The enthalpically costly conformational transition is "paid for" by the enthalpy that would have otherwise been realized through formation of bonding interactions between the protein and ligand. The entropic switch from highly unfavorable for weak effectors to modestly favorable for strong effectors may indicate that allosteric activation for dimerization requires increased protein flexibility. Alternatively, this switch may reflect solvent-based phenomena that are coupled to the allosteric activation process.

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