Thermodynamic Analysis of Small Ligand Binding to the *Escherichia coli* Repressor of Biotin Biosynthesis†

Yan Xu,‡ Craig R. Johnson,§ and Dorothy Beckett*,‡

Department of Chemistry and Biochemistry, University of Maryland Baltimore County, Baltimore, Maryland 21228, and Department of Biology and Biocalorimetry Center, The Johns Hopkins University, Baltimore, Maryland 21218

Received January 10, 1996; Revised Manuscript Received February 29, 1996⊗

ABSTRACT: BirA is the transcriptional repressor of biotin biosynthesis and a biotin holoenzyme synthetase. It catalyzes synthesis of biotinyl-5′-AMP from the substrates biotin and ATP. The adenylate is the activated intermediate in the biotin transfer reaction as well as the positive allosteric effector for site-specific DNA binding. The affinity of BirA for the adenylate is considerably greater than its affinity for biotin, and both binding reactions are coupled to changes in the conformation of the protein. The temperature dependencies of the two binding interactions have been determined using kinetic techniques.Van't Hoff analyses of the equilibrium dissociation constants derived from the kinetic data indicate that while the two binding processes are characterized by large negative enthalpies, the entropic contributions are small for both. Binding enthalpies have also been determined by isothermal titration calorimetry. Consistent with the results of the van't Hoff analyses, the calorimetric enthalpies are large and negative. The greater precision of the calorimetric measurements allowed more accurate estimation of the entropic contributions to the binding processes, which are of opposite sign for the two ligands. In addition, the heat capacity changes associated with the two binding reactions are small. The measured thermodynamic parameters for binding of biotin and bio-5′-AMP to BirA have been utilized to dissect out structural contributions to the binding energetics. Results of these calculations indicate equivalent contributions of burial of polar and apolar surface area to both binding processes. The total loss of solvent accessible surface area is, however, greater for biotin binding. The analysis indicates furthermore that although both binding reactions are coupled to losses in configurational entropy, the magnitude of the conformational change is significantly larger for biotin binding.

The 35.3 kDa repressor of biotin biosynthesis is a multifunctional protein that plays a central role in both retention of biotin and regulation of its synthesis in *Escherichia coli* (Barker & Campbell, 1981a; Eisenberg et al., 1982). The protein catalyzes synthesis of biotinyl-5′-AMP (bio-5′-AMP) from the two substrates, biotin and ATP (Prakash & Eisenberg, 1979). The BirA−adenylate complex participates in two processes. It binds to the biotin carboxyl carrier protein (BCCP) subunit of the acetyl-CoA carboxylase which results in BirA-catalyzed transfer of biotin from the adenylate to a unique lysine residue on BCCP (Barker & Campbell, 1981a; Eisenberg et al., 1982). Alternatively, the BirA−adenylate complex binds to the 40 base pair biotin operator sequence to repress transcription of the biotin biosynthetic operon (Prakash & Eisenberg, 1979; Barker & Campbell, 1981b). The switch in BirA function from biotin ligase to transcriptional repressor is regulated by the intracellular concentration of the unbiotinated or apoBCCP (Cronan, 1989). At high intracellular concentrations of this protein the BirA−bio-5′-AMP complex favorably partitions toward the biotin ligase function. Upon depletion of the intracellular apoBCCP pool, the BirA−adenylate complex accumulates to a sufficient level to saturate the biotin operator and repress transcription of the genes that code for the biotin biosynthetic enzymes. In this system the adenylate serves as both the activated intermediate in biotin transfer and as the positive allosteric effector in site-specific DNA binding. Adenylate binding is, therefore, critical for regulating the functional state of BirA.

Studies of binding of both biotin and bio-5′-AMP reveal that both protein−ligand complexes are very stable. The Gibbs free energy for binding of biotin is −10 kcal/mol in buffer containing 10 mM Tris HCl, pH 7.50 ± 0.02, at 20 °C, 200 mM KCl, and 2.5 mM $MgCl_2$, while that for adenylate binding is −14 kcal/mol (Xu et al., !995). Kinetic measurement of binding of the two ligands as well as results of partial proteolytic digestion of apoBirA and its complexes with biotin and bio-5′-AMP indicate that conformational transitions occur concomitant with ligand binding (Xu et al., 1995). The nature of the conformational transition is, moreover, distinct for the two ligands. Although the molecular basis for the difference in the energetics of binding of BirA to biotin and bio-5′-AMP is unknown, it must partially reflect differences in the energetics of the conformational transition that is coupled to each process. Regulation of function in BirA is likely to be intimately linked to the ligand-induced conformational transitions, and elucidation of the thermodynamic driving forces for biotin and bio-5′-AMP binding to BirA will contribute to a more complete understanding of functional switching in this protein.

The structure of BirA has been determined by X-ray crystallography (Wilson et al., 1992). No structures of complexes with biotin and bio-5′-AMP are yet available, and diffusion of these ligands into crystals of apoBirA results in

† Supported by NIH Grant GM46511 and a DuPont Young Professorship.

* Author to whom correspondence should be addressed.
‡ University of Maryland Baltimore County.
§ The John Hopkins University.

⊗ Abstract published in *Advance ACS Abstracts,* April 1, 1996.

S0006-2960(96)00065-7 CCC: $12.00

severe cracking of the crystals. This observation is consistent with solution evidence for the occurrence of conformational changes in the protein upon ligand binding. A structure of BirA bound to biotinyllysine, an analog for the product of the biotin ligation reaction, has been determined (Wilson et al., 1992). The ligand binding site is found in a region of the three-dimensional structure that contains two disordered segments of the polypeptide chain composed of residues 116−124 and 212−223. Residues 116−118 are ordered in the cocrystal structure. Results of partial proteolytic digestion of complexes of BirA with biotin and bio-5′-AMP suggest that the magnitude of the disorder to order transition that occurs upon binding of these ligands is more substantial than that observed in the available structures (Xu et al., !995). Results of a large number of thermodynamic studies of protein−ligand interactions reveal that binding is frequently accompanied by a large negative heat capacity change (ΔC_p) (Sturtevant, 1977; Spolar & Record, 1994). These changes reflect the burial of polar and apolar surface area either by direct desolvation at the binding interface or through conformational changes coupled to the binding process (Murphy et al., 1992, 1993, 1994; Spolar & Record, 1992, 1994; Gomez et al., 1995). Thermodynamic studies of binding of BirA to biotin and bio-5′-AMP are, therefore, of particular interest in light of the indications that both binding reactions are coupled to disorder to order transitions in the polypeptide chain.

In this work we have used two experimental approaches to determine the thermodynamics of interaction of BirA with bio-5′-AMP and biotin. Kinetic methods have been used to measure the temperature dependencies of both the bimolecular association of BirA with each ligand as well as the unimolecular dissociation rates of the complexes. Results of these measurements indicate that both the association and dissociation rate constants increase with increasing temperature. The magnitudes of the activation energies obtained from Arrhenius analysis of the dissociation data are large; 22 kcal/mol for BirA−biotin and 18 kcal/mol for BirA−bio-5′-AMP. The rate data were used to calculate equilibrium dissociation constants for the binding reactions as a function of temperature. Van't Hoff analyses of the resulting parameters reveal that the Gibbs free energy of binding remains roughly constant over the temperature range of the study. Both reactions were found to be driven by large negative enthalpies. Direct calorimetric measurements of the binding enthalpies were also performed. While the results of these measurements support the conclusion based on van't Hoff analyses that both reactions are driven primarily by large favorable enthalpies, the greater precision of the calorimetric measurements allows for more accurate assessment of the entropic contribution to binding. The value of the entropic contribution, $-T\Delta S$, to binding of BirA to bio-5′-AMP is −6 kcal/mol, while that for the BirA-biotin binding is +6 kcal/mol at 20 °C. Analysis of the dependence of the binding enthalpy on temperature reveals that both binding processes are accompanied by moderate negative heat capacity changes. Structural features of the binding processes were calculated using the measured thermodynamic parameters. Results of these calculations indicate that although both binding reactions are accompanied by burial of roughly equivalent amounts of polar and apolar surface area, the magnitude of the change in accessible surface area is approximately 1.5-fold greater for biotin than for bio-5′-AMP binding to BirA. The calculations reveal, moreover, unfavorable contributions due to the loss of configurational entropy to the energetics of both binding processes. The magnitude of the change is, however, much greater for biotin binding and is a major source of the large unfavorable entropy associated with this binding process.

MATERIALS AND METHODS

Chemicals and Biochemicals. All chemicals used in preparation of buffers were at least reagent grade. *d*-Biotin was purchased from Sigma. Bio-5′-AMP was synthesized and purified using a modification of the procedure described in Lane et al. (1965) (Abbott & Beckett, 1993). BirA was purified as described in Abbott and Beckett (1993).

Stopped-Flow Fluorescence Measurements. All stopped-flow fluorescence measurements were made using a KinTek stopped-flow instrument (Model 2001). Experiments were performed in buffer A (10 mM Tris-HCl, pH 7.50 ± 0.02, 200 mM KCl, 2.5 mM $MgCl_2$). The pH of each buffer was adjusted at the working temperature. Buffers were degassed by bubbling helium through them, and solutions were filtered through 0.45-μm Acrodisc PTFE filters (Gelman Sciences) before use. The excitation wavelength was set at 295 nm for all measurements, and fluorescence emission was monitored above 340 nm using a cutoff filter (Corion Corp.). Constant temperature was maintained by circulating water from a constant temperature bath through the stopped-flow module.

Measurement of the Rate of Association of BirA with Biotin and Bio-5′-AMP. The rates of association of biotin and bio-5′-AMP with BirA were measured by stopped-flow fluorescence. Equal volumes of the protein and ligand were rapidly mixed, and the resultant time-dependent change in fluorescence was measured. The protein concentration was constant for all measurements while the ligand concentrations were varied. All measurements were performed in buffer A .

Measurement of the Rate of Dissociation of the BirA−Biotin Complex. The unimolecular rate of dissociation of the BirA−biotin complex was measured by stopped-flow fluorescence. A 1:1 complex of the protein and ligand was rapidly combined with a large molar excess of the competing ligand, bio-5′-AMP, and the resulting time-dependent decrease in the intrinsic BirA fluorescence was measured.

Measurement of the Dissociation Rate of the BirA−Bio-5′-AMP Complex. The unimolecular rate of dissociation of the BirA−bio-5′-AMP complex was measured using the method described in Xu and Beckett (1994). The method involves measurement of the time course of BirA-catalyzed synthesis of bio-5′-AMP from the substrates biotin and ATP. The time course of bio-5′-AMP synthesis was monitored by quantitation of the amount of product, bio-5′-AMP, formed as a function of time. Reaction mixtures contained [α-^{32}P]-ATP (approximately 5 000 000 cpm), BirA, ATP, and biotin in buffer A (adjusted to pH 7.50 ± 0.02 at the working temperature). The concentrations of components present in the reported measurements are indicated in Results. In order to drive the reaction equilibrium toward completion, inorganic pyrophosphatase (Boehringer Mannheim) that had been dialyzed against buffer A was also present at a final concentration of 2 units/mL. Reactions were initiated by addition of biotin to mixtures containing all other reaction components and were incubated at the appropriate temper-

ature. Time points were obtained by quenching 3 μL aliquots of the reaction mixtures into 1 μL of a large molar excess (4 mM) of chemically synthesized cold bio-5′-AMP. A 1 μL aliquot of each quenched time point was spotted onto a cellulose TLC plate (Kodak Co.). The product [^{32}P]bio-5′-AMP was resolved from the reactant [α-^{32}P]ATP by chromatography using a mobile phase containing H_2O/formic acid/tert-amyl alcohol (1/2/3 v/v/v). Quantitation of the bio-5′-AMP and ATP was performed using the Molecular Dynamics PhosphorImager System. Phosphor screens were exposed for approximately 5 h prior to scanning. Radioactivity present in the spots corresponding to ATP and bio-5′-AMP was quantitated at each time point. Bio-5′-AMP concentration was calculated by dividing the activity in the bio-5′-AMP spot by the total sum of the activity in the two spots and multiplying the result by the initial ATP concentration.

Calorimetric Measurements. Calorimetry was performed in a MicroCal Omega 2 isothermal titration calorimeter (Biocalorimetry Center, The Johns Hopkins University). In all measurements protein was loaded into the cell, and the ligand, either biotin or bio-5′-AMP, served as the titrant. The protein was dialyzed extensively against the appropriate buffer before use. Biotin solutions were prepared in the same buffer, and a concentrated solution of bio-5′-AMP (in H_2O) was diluted into the buffer immediately before use. All solutions were properly degassed. Two types of experiments were performed. The total heats of binding of biotin and bio-5′-AMP to BirA were determined by addition of an excess of ligand required to fully saturate the protein. The equilibrium dissociation constants previously measured for the interaction of BirA with biotin and bio-5′-AMP in buffer A at 20 °C are 4×10^{-8} and 4×10^{-10} M, respectively (Xu et al., 1995). These high affinities ensure that stoichiometric binding conditions are obtained in the calorimetric measurements of the total heats of binding. The total heats were measured in both phosphate and Tris-HCl buffers to determine if any protonation or deprotonation events contribute to the binding at pH 7.5. Total heats of binding were also measured over a range of temperatures in order to estimate the heat capacity change associated with each binding process. A complete binding curve for the interaction of BirA with biotin was measured to obtain independent measures of the equilibrium constant and enthalpy change for the binding process. The high affinity of BirA for bio-5′-AMP precluded determination of the equilibrium constant for this binding process using isothermal titration calorimetry. The ITCArea program (MicroCal) was used for numerical integration of the heat flow data obtained in each measurements. This program allows for linear compensation of baseline drifts by extrapolation of the pre- and post-peak baselines. The resulting series of heats obtained from the full titration data were used directly in nonlinear least-squares fitting. Heats of dilution of the ligand were determined for both types of calorimetric measurements and were appropriately subtracted from the measured heats of ligand binding.

Data Analysis. Nonlinear least-squares analysis of the primary stopped-flow fluorescence data was performed using the software provided with the instrument. The resulting parameters obtained from these analyses were used to obtain the microscopic rate constants for binding of BirA to biotin and bio-5′-AMP. These rate constants were estimated using weighted linear least-squares methods. The weights used in the least-squares analysis included both the standard errors in the parameters obtained from nonlinear least-squares analysis of the primary data as well as the standard error of the mean of these parameters obtained from analysis of several data sets. The resulting errors in these microscopic rate parameters were propagated into the calculated equilibrium binding constants using the methods described in Taylor (1982).

RESULTS

We have previously shown that the bimolecular association of both biotin and bio-5′-AMP with BirA occurs in two steps (Xu et al., 1995). The data have been interpreted to indicate initial rapid formation of a collision complex followed by a slow conformational change in the complex. The following general two-step binding model is consistent with this interpretation:

$$\text{BirA} + \text{ligand} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{BirA}-\text{ligand} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} *\text{BirA}-\text{ligand} \quad (1)$$

where BirA−ligand represents the collision complex and *BirA−ligand is the final form of the complex. Representative stopped-flow fluorescence traces obtained for measurement of association of BirA with bio-5′-AMP and biotin are shown in Figure 1A. The data are well described by the following double-exponential equation:

$$F = A_1 \exp(1/\tau_1 t) + A_2 \exp(1/\tau_2 t) + C \quad (2)$$

where F is the fluorescence intensity, A_1 and τ_1 and A_2 and τ_2, are the amplitudes and the relaxation times for the first and second phases, respectively, and C is the baseline fluorescence signal of the BirA−ligand complex. The following equation relates the measured time constants for the two kinetic phases to the microscopic rate parameters governing the binding process (Fersht, 1985):

$$\frac{1}{\tau_1} + \frac{1}{\tau_2} = k_1[\text{ligand}] + k_{-1} + k_2 + k_{-2} \quad (3)$$

At the high concentrations of ligand and protein utilized in our measurements, $1/\tau_2 = k_2 + k_{-2}$ (Xu et al., 1995) and eq 3 simplifies to $1/\tau_1 = k_1[\text{ligand}] + k_{-1}$. Analysis of the linear dependence of $1/\tau_1$ on ligand concentration yields k_1 as the slope and k_{-1} as the intercept. We have previously shown that for binding of both biotin and bio-5′-AMP to BirA, k_1 provides an accurate estimate of k_{on} (Xu et al., 1995), the apparent second order rate constant governing formation of the BirA−ligand complex. We have used this approximation for analysis of kinetic data obtained over the temperature range from 15 to 35 °C. Results of the analysis for association of bio-5′-AMP with BirA are shown in Figure 1B. Values of k_1 were obtained over the temperature range of 10−30 °C for biotin binding and 15−35 °C for bio-5′-AMP binding to BirA. These data were used to determine the activation energies for the association processes from an Arrhenius analysis. Results of these analyses are shown in Figure 2. The activation energies for the bimolecular association reactions are similar for binding of the two ligands to BirA (Table 1).

Temperature Dependence of the Rates of Dissociation of the BirA−Bio-5′-AMP and BirA−Biotin Complexes. The

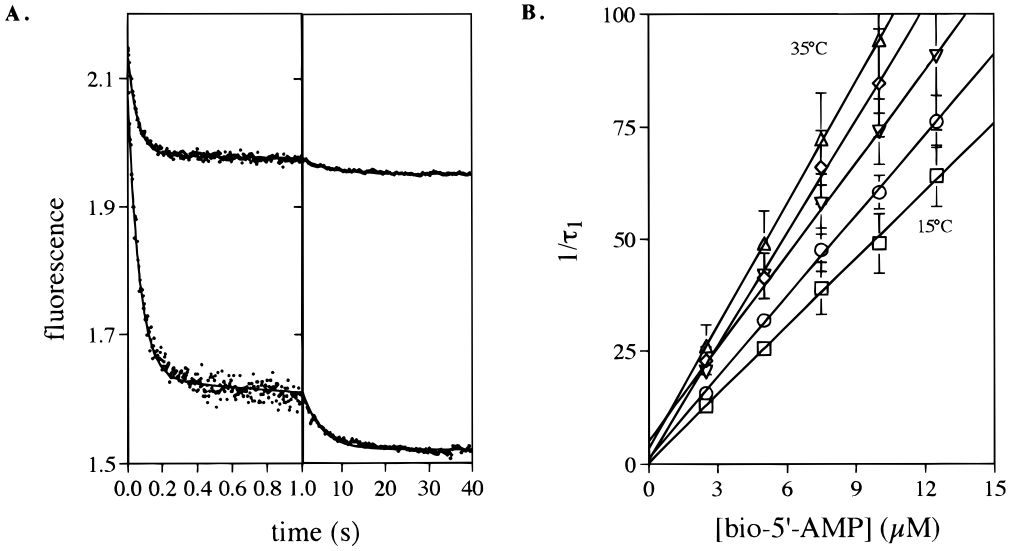

FIGURE 1: (A) Time courses for association of BirA with biotin (upper trace) and bio-5′-AMP (lower trace) obtained by stopped-flow fluorescence. Measurements were made in buffer A (10 mM Tris-HCl, pH 7.50 ± 0.02, 200 mM KCl, 2.5 mM $MgCl_2$ at 20 °C). The two panels represent data acquired on different time scales. [BirA] = 0.5 μM, [bio-5′-AMP] = 2.5 μM, [biotin] = 2.5 μM. (B) Temperature dependence of the ligand concentration dependence of the first phase in association of BirA with bio-5′-AMP. The solid lines were simulated using the best fit parameters obtained from weighted linear least-squares analysis of the apparent rate, $1/\tau_1$, versus [bio-5′-AMP] data.

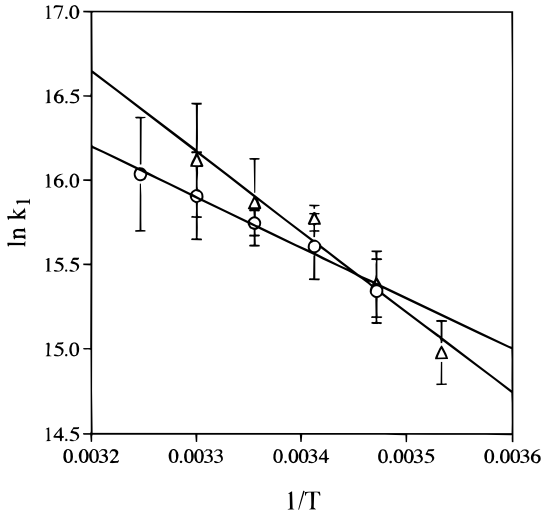

FIGURE 2: Arrhenius analysis of the association rate data obtained for binding of biotin (△) and bio-5′-AMP (○) to BirA. The solid lines were simulated using the best fit parameters obtained from weighted linear least-squares analysis of the k_1 versus $1/T$ data.

Table 1: Arrhenius Activation Energies[a] Governing Association and Dissociation of the BirA−Ligand Complexes

	association (kcal/mol)	dissociation (kcal/mol)
bio-5′-AMP	6 ± 4	18 ± 4
biotin	9 ± 4	23.4 ± 0.1

[a] Activation energies were calculated using the Arrhenius equation: $\ln k = -E_a/RT + \ln A$, where k is the rate constant (either the apparent second-order assocation rate constant or the first-order dissociation rate constant), E_a is the apparent activation energy for the process, A is the pre-exponential factor, and R is the gas constant.

unimolecular rate of dissociation of the BirA−bio-5′-AMP complex could not be measured using the change in intrinsic protein fluorescence as the observable. This is due to the small value of the rate constant (0.00027 s^{-1}at 20 °C) coupled with photobleaching of the intrinsic BirA fluorescence signal over long time periods. We have developed an alternative method to determine the dissociation rate which involves measurement of the time course of BirA-catalyzed synthesis of bio-5′-AMP from the substrates biotin and ATP (Xu & Beckett, 1994). The time course is characterized by two phases: an initial rapid exponential burst of synthesis of 1 mol of adenylate per mol of enzyme followed by a slow linear phase, the rate of which is limited by the release of product, bio-5′-AMP, from the enzyme. The slope of the slow linear phase can be used to obtain the rate of dissociation of the complex using the following expression:

$$[\text{bio-5-AMP}]_t = n[\text{BirA}]_0 + n[\text{BirA}]_0 k_{\text{off}} t \qquad (4)$$

where [bio-5′-AMP] is the concentration of the adenylate at time = t, n is the stoichiometry of the burst complex, $[\text{BirA}]_o$ is the total BirA concentration, and k_{off} is the unimolecular dissociation rate constant of the complex. Provided that the substrates are present in great excess over the enzyme, the slope of the [bio-5′-AMP]/$[\text{BirA}]_o$ vs time plot can be used to obtain the value of k_{off}. Results of measurements performed over a temperature range from 15−40 °C are shown in Figure 3. The dissociation rate of the BirA−bio-5′-AMP increases approximately 10-fold over the temperature range of the measurements. The Arrhenius plot obtained from these data is shown in Figure 4. The activation energy for the dissociation process estimated from the analysis is large (Table 1).

Temperature Dependence of the Rate of Dissociation of the BirA−Biotin Complex. The rate of dissociation of the BirA−biotin complex was measured by stopped-flow fluorescence as described in Xu et. al. (1995). A 1:1 complex of BirA and biotin was rapidly mixed with a large molar excess of bio-5′-AMP, and the resulting time-dependent decrease in intrinsic BirA fluorescence was monitored. The process is well described by a single-exponential equation over the entire temperature range from 10 to 35 °C. An Arrhenius plot obtained from analysis of the data is shown in Figure 4. The activation energy for the dissociation of the BirA−biotin complex is, like the BirA−bio-5′-AMP complex, large (Table 1).

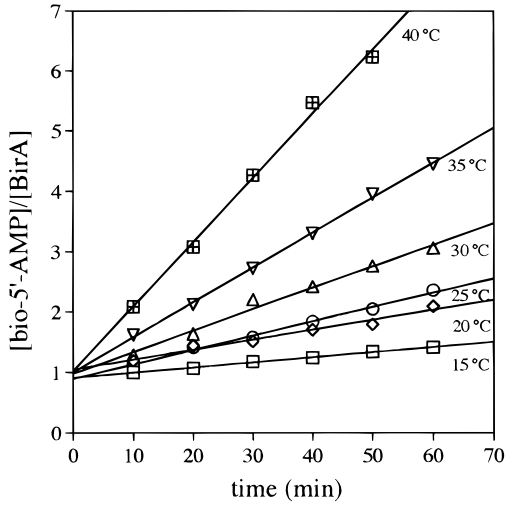

FIGURE 3: Dependence of the linear phase of the time course in BirA-catalyzed synthesis of bio-5′-AMP on temperature. The solid lines were obtained from linear least-squares analysis of the data at each temperature. In each measurements [BirA] = 1 μM , [biotin] = 100 μM, [ATP] = 500 μM. All measurements were performed in buffer A titrated to pH 7.50 ± 0.02 at the working temperature.

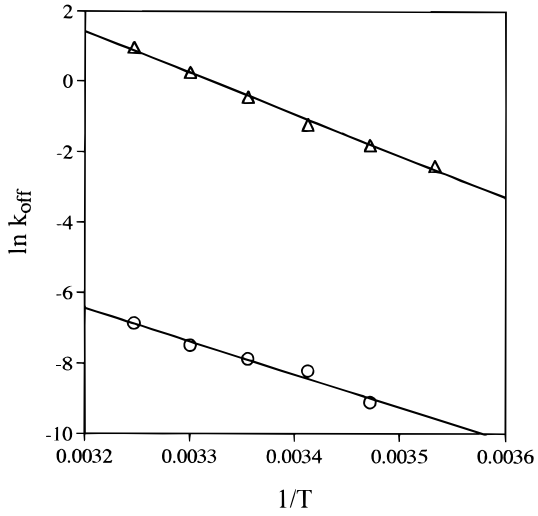

FIGURE 4: Arrhenius analysis of the dissociation rate data obtained for the BirA−biotin (△) and BirA-bio-5′-AMP (○) complexes as a function of temperature. The solid lines were simulated using parameters obtained from weighted linear least squares analysis of the data.

Temperature Dependence of the Equilibrium Dissociation Constants for Binding of Biotin and Bio-5′-AMP to BirA. The association and dissociation rate data described above were used to estimate the equilibrium dissociation constants for binding of BirA to the two ligands. In general, for a two-step binding reaction like that shown in eq 1, the equilibrium dissociation constant is related to the microscopic rate constants governing binding by the expression $K_D = k_{-1}k_{-2}/k_1k_2$. We have, however, previously shown that k_{-1} is approximately equal to k_2 for binding of both biotin and bio-5′-AMP to BirA (Xu et al., 1995). In addition, k_{off}, the overall dissociation rate constant for both complexes is equal to k_{-2}. The equilibrium dissociation constant for binding of the two ligands to BirA can, therefore, be expressed in terms of the microscopic rate constants using the expression $K_D = k_{off}/k_1$. The temperature dependencies of the equilibrium parameters were analyzed using the van't Hoff formalism (Figure 5) to estimate the enthalpic and entropic

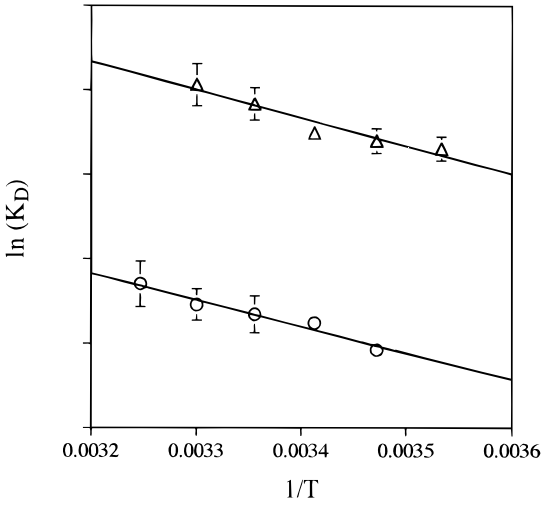

FIGURE 5: Van't Hoff analysis of the temperature dependence of the equilibrium dissociation constants for binding of BirA to biotin (△) and bio-5′-AMP (○). Equilibrium dissociation constants were calculated from the rate parameters using the familiar equation $K_D = k_{off}/k_{on}$ where $k_{on} = k_1$ (see Results).

Table 2: Thermodynamic Parameters for Binding of BirA to Biotin and Bio-5′-AMP Obtained from Van't Hoff Analyses[a]

	ΔH (kcal/mol)	$-T\Delta S$ (kcal/mol)[b]
bio-5′-AMP	−12 ± 3	0 ± 2
biotin	−13 ± 4	2 ± 3

[a] Equilibrium constants used in the van't Hoff analyses were calculated from the rate data using the familiar equation $K_D = k_{off}/k_{on}$ where $k_{on} = k_1$ in eq 1. [b] Calculated at 20 °C (293 K).

contributions to the two binding processes (Table 2). The errors in these values are estimated using the extremes of the confidence intervals to calculate the maximum and minimum slopes and intercepts for the linear plots. While the equilibrium dissociation constants for both binding reactions increase approximately 10-fold over the temperature range studied, very little variation is observed in the Gibbs free energies of binding calculated over this same temperature range. The van't Hoff analyses of the binding data for both ligands indicate that the reactions are enthalpically driven. The entropic contribution to the binding process is small for both ligands and the linearity of the plots indicates that heat capacity change for both binding processes is small. Given the errors associated with the van't Hoff analyses, however, we cannot be absolutely certain of this conclusion.

Calorimetric Measurements of the Enthalpies and Heat Capacities of Ligand Binding to BirA. Direct calorimetric measurements were performed in order to obtain independent estimates of the thermodynamic parameters governing binding of BirA to biotin and bio-5′-AMP. The total heats of binding of both ligands to BirA were measured by titrating a molar excess of ligand into a protein solution. Multiple measurements of the heats of dilution of the same quantity of ligand were performed and the values subtracted accordingly. Previous studies indicate that, although there is no evidence for assembly of monomers of apoBirA or the BirA−biotin complex, bio-5′-AMP binding is positively linked to dimerization of BirA and the equilibrium dissociation constant for assembly of BirA−bio-5′-AMP in buffer A is approximately 40 μM (Eisenstein and Beckett, unpublished results). All calorimetric measurements of bio-5′-AMP binding were therefore performed at a total protein

Table 3: Results of Calorimetric Measurements of Binding of Biotin and Bio-5′-AMP to BirA

ligand	buffer	temperature (°C)	ΔH (kcal/mol)	ΔC_p (cal/mol/K)
biotin	phosphate[a]	19.1	-15.5 ± 0.5	-160 ± 40
	Tris-HCl[b]	19.1	-13.0 ± 0.2	
bio-5′-AMP	phosphate	21.7	-8.1 ± 0.6	-150 ± 50
	Tris-HCl	19.1	-7.3 ± 0.2	

[a] Measured in 10 mM potassium phosphate, pH 7.50 ± 0.02, 200 mM KCl, and 2.5 mM $MgCl_2$. [b] Measured in 10 mM Tris-HCl, pH 7.50 ± 0.02, at 20.0 ± 0.1 °C, 200 mM KCl, and 2.5 mM $MgCl_2$.

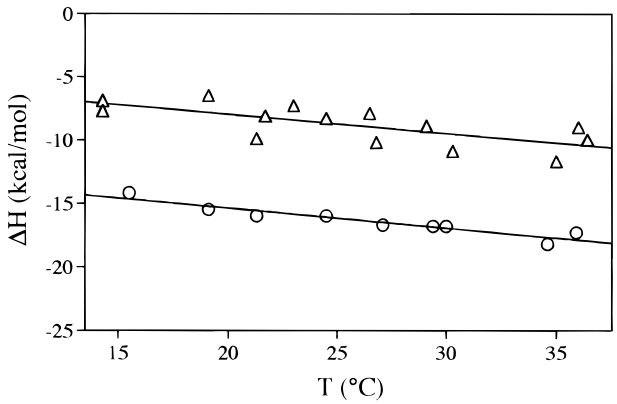

FIGURE 6: Dependence of the enthalpies of binding of BirA to biotin (△) and bio-5′-AMP (○) on temperature. Measurements were performed by isothermal titration calorimetry as described in the text. The solid lines were simulated using the best fit parameters obtained from linear least-squares analysis of the data.

concentration between 2.5 and 3 μM to ensure that the protein was monomeric. The total heats of binding measurements were performed both in Buffer A and in 10 mM potassium phosphate, pH 7.50 ± 0.02, 200 mM KCl, and 2.5 mM $MgCl_2$, in order to determine if protonation events contribute to either binding process at this pH. Results of these experiments indicate that while there may be a small contribution of proton binding to the heat of binding of biotin, no protonation event is linked to bio-5′-AMP binding. The total enthalpies of binding obtained at approximately 20 °C in both buffers are shown in Table 3. The heat capacity changes for binding of biotin and bio-5′-AMP to BirA were estimated from the temperature dependence of the total heat of binding obtained over the range of 15−35 °C. Results of these analyses are shown in Figure 6. As indicated in the figure, the errors associated with the determinations of the enthalpies of bio-5′-AMP binding are significantly larger than those associated with biotin binding. This is due to the limitation in the total protein concentration that could be used for measurements of adenylate binding and the resulting small total measured heats which were on the order of 30−50 μcal. The heat capacities determined from the slopes are shown in Table 3. These moderate heat capacity changes are consistent with the results of the van't Hoff analyses of the equilibrium parameters calculated from kinetic data.

The Gibbs free energy for binding of bio-5′-AMP to BirA could not be determined by direct calorimetric measurements because the equilibrium dissociation constant is in the subnanomolar range of concentration. Direct calorimetric measurement of binding of biotin to the protein could, however, be made since the K_D for this interaction in buffer A is approximately 8×10^{-8} M at 25 °C. The thermogram

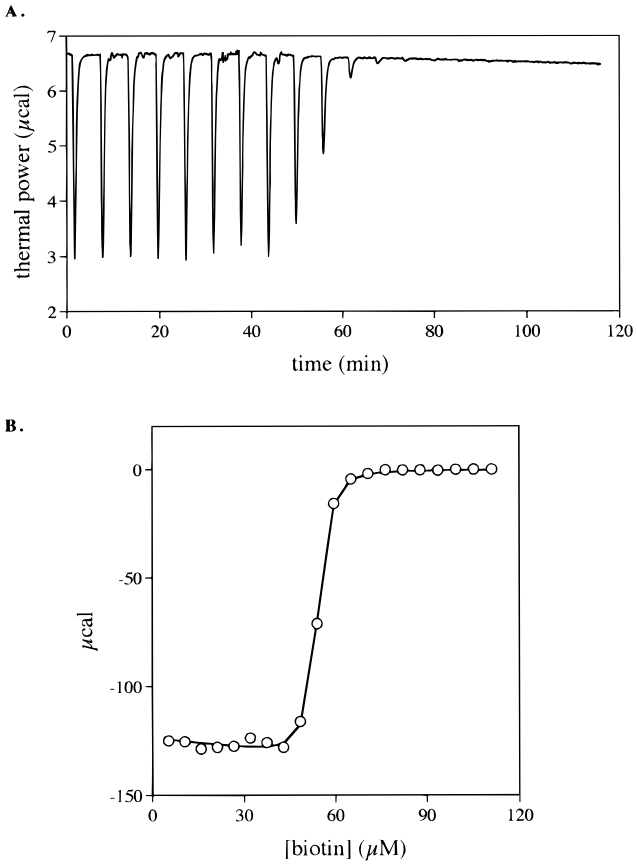

FIGURE 7: (A) Calorimetric titration of BirA with biotin. The titration consists of 20 10 μL injections of a 707 μM biotin stock solution. The biotin solution was injected into a sample cell containing 50.5 μM BirA (volume = 1.362 mL) at a temperature of 24.6 °C. Buffer: 10 mM potassium phophate, pH 7.50 ± 0.02, 200 mM KCl, and 2.5 mM $MgCl_2$. (B) Integrated calorimetric heats (data points) and best fit curve to a simple single-site binding model.

obtained for a complete titration of BirA with biotin is shown in Figure 7A. Analysis of the data using a single-site binding model is shown in Figure 7B. The high affinity of BirA for biotin places this reaction at the limit of determination of a equilibrium constant by isothermal titration calorimetry. In analyzing the binding data, the enthalpy of binding was, therefore, fixed at the value obtained from measurements of the total heat of binding. The equilibrium dissociation constant for the binding process obtained from the analysis is 8.9×10^{-8} M, which is in excellent agreement with the value calculated from the kinetic data in similar buffer conditions.

DISCUSSION

In this study we have used two approaches in determining the thermodynamics of binding of BirA to the two ligands, biotin and bio-5′-AMP. Biotin is a substrate in BirA-catalyzed synthesis of bio-5′-AMP. The adenylate serves both as the activated intermediate in transfer of biotin to BCCP and as the corepressor in site-specific DNA binding to the biotin operator sequence. BirA−bio-5′-AMP is, thus, the "active" form of BirA for both its biotin ligase and transcriptional repressor functions. We have previously shown that both ligands bind with high affinity to BirA. The Gibbs free energy for binding of bio-5′-AMP to BirA is −4 kcal/mol more favorable than that for binding of biotin in buffer A at 20 °C (Xu et al., 1995). We have, moreover,

Table 4: Dissection of the Thermodynamic Contributions to the Dissociation Kinetics of the BirA−Biotin and BirA−Bio-5′-AMP Complexes

ligand	$\Delta G^{\ddagger}$ [a] (kcal/mol)	$\Delta H^{\ddagger}$ (kcal/mol)	$-T\Delta S^{\ddagger}$ [a] (kcal/mol)
bio-5′-AMP	22.0 ± 0.1	18 ± 2	4 ± 1
biotin	17.7 ± 0.1	22.9 ± 0.8	−5.1 ± 0.5

[a] The transition state free energies and the entropic contributions were calculated at 20 °C.

obtained evidence for the occurrence of conformational changes in BirA upon binding of both ligands. The goal of the present study is to determine the thermodynamic driving forces for these two highly specific binding interactions. Results of these studies may provide insights into the mechanism of interconversion of BirA from one functional state to another.

Temperature Dependence of the Kinetics of Binding of Biotin and Bio-5′-AMP to BirA. We have used a combination of direct kinetic measurements of binding by stopped-flow fluorescence and measurements of the rates of BirA-catalyzed synthesis of bio-5′-AMP to determine the temperature dependence of the kinetics of binding of the two ligands to BirA. Results of these measurements indicate that the bimolecular rates of association and the unimolecular rates of dissociation increase with increasing temperature for both reactions. The activation barriers for dissociation of the two complexes are large. Interestingly, dissociation of the BirA−biotin complex, which is 1000-fold faster than dissociation of the BirA−bio-5′-AMP complex at 20 °C, has a greater activation energy associated with it. The Arrhenius activation energy, E_a, is approximately equivalent to the transition state enthalpy for a kinetic process (Jencks, 1987). We have previously shown that the kinetic basis for the difference in affinities of BirA for biotin and bio-5′-AMP is primarily a difference in the magnitudes of k_{-2} for the two processes (Xu et al., 1995). k_{-2} is the first-order rate constant governing isomerization of the final protein−ligand complex to the intermediate or collision complex. Analysis of the temperature dependence of the dissociation kinetics of the two complexes may prove useful for understanding the structural basis for the difference in kinetic stabilities of the two complexes. The transition state free energies, $\Delta G^{\ddagger}$, for dissociation of the BirA−ligand complexes can be calculated at any temperature using the following equation [see, for example, Jencks (1987)]:

$$\Delta G^{\ddagger} = -RT \ln\left(\frac{k_{off} h}{k_B T}\right) \quad (5)$$

where R is the gas constant, k_{off} is the unimolecular dissociation constant, h is Planck's constant, and k_B is the Boltzmann constant. The transition state free energy can be partitioned into enthalpic and entropic contributions using the following relationship (Jencks, 1987):

$$\ln\left(\frac{k_{off}}{T}\right) = -\frac{\Delta H^{\ddagger}}{R}\left(\frac{1}{T}\right) + \frac{\Delta S^{\ddagger}}{R} + \ln\left(\frac{k_B}{h}\right) \quad (6)$$

where $\Delta H^{\ddagger}$ is the transition state enthalpy, $\Delta S^{\ddagger}$ is the transition state entropy, and all other symbols are defined above. We have analyzed the dissociation data using eq 6, and results of the analysis are shown Table 4. The difference in the magnitudes of the transition state free energies for release of the two ligands reflects the slower rate of dissociation of the BirA−bio-5′-AMP complex. Results of the analysis further indicate that although the magnitude of the transition state enthalpy is large for the BirA−biotin complex, it is offset by a favorable entropic contribution. Both the entropic and enthalpic contributions to the transition state free energy for dissociation of BirA−bio-5′-AMP are positive. In dissociation of both complexes the enthalpic contribution to the transition state free energy is significantly larger than the entropic contribution. The transition state enthalpies associated with dissociation most likely reflect disruption of noncovalent bonds in the course of conversion of the bound complex to the transition state. The large magnitudes of the enthalpic components of the transition state free energies are consistent with disruption of hydrogen bonding and van der Waals interactions (Ross & Subramanian, 1981). Entropic contributions to the transition state free energies are of opposite sign for the two ligands. While interpretation of the difference in entropic contributions to kinetic barriers is complicated by the unknown role of solvent in the process (Jencks, 1987), the results may reflect the conformational flexibilities of the transition state relative to the final form of the complex. The positive transition state entropy associated with isomerization of the BirA−biotin complex suggests an increase in disorder upon conversion of the final complex to the transition state. The opposite is true for the BirA−adenylate complex.

Van't Hoff Analysis of the BirA−Ligand Interactions. The temperature dependence of the kinetics of binding of BirA to biotin and bio-5′-AMP have been used to calculate the equilibrium dissociation constants for the reactions over the temperature range studied. Only small changes are observed in the Gibbs free energies for binding of both ligands over the temperature range studied. Values of the enthalpies and entropies of binding of BirA to the two ligands were obtained from van't Hoff analysis of the data. Results of these analyses indicate that both binding process have large negative enthalpies associated with them. The entropic contribution to binding is, within error, negligable for both ligands.

Calorimetric Measurements of Binding of BirA to Biotin and Bio-5′-AMP. Direct calorimetric measurements of binding of BirA to the two ligands were performed, and results of these measurements agree, within error, with the parameters obtained from analysis of kinetic data. The calorimetrically measured heats of binding are consistent with the results of van't Hoff analysis in indicating large favorable enthalpic components to both binding processes. The calorimetric results indicate, however, that the magnitude of the enthalpic contribution is considerably greater for biotin than for bio-5′-AMP. This information could not be extracted from the van't Hoff analyses due to the inherently large errors associated with this type of analysis. The moderate dependence of the binding enthalpy on buffer indicates that at pH 7.5 the contributions of protonation events to the two binding processes are small. Additional measurements must be performed over a broad pH range to further investigate the role of protonation events in the two binding reaction. The precision of the calorimetric data allows more accurate estimation of the entropic contributions to the binding processes. At 20 °C the entropic contribution to the binding free energy for the bio-5′-AMP−BirA

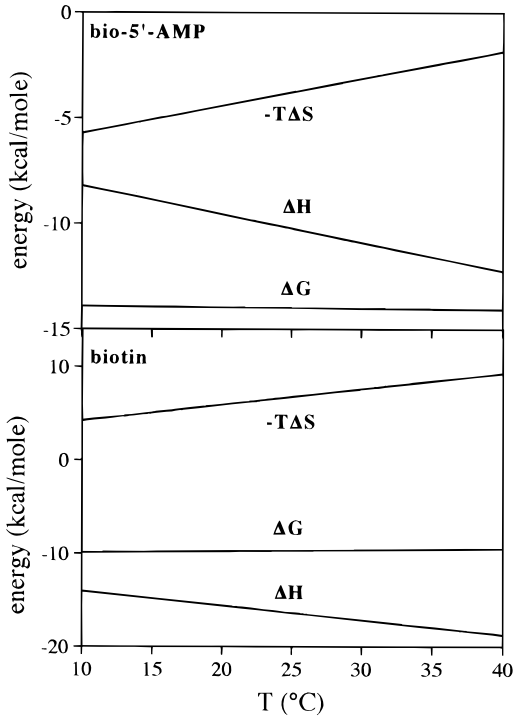

FIGURE 8: Thermodynamic profiles for binding of bio-5′-AMP and biotin to BirA based on the combined results of calorimetric measurements of the heats of binding and the equilibrium dissociation constants for binding calculated from kinetic parameters. The values of ΔC_p are assumed to be constant over the temperature range.

interaction is approximately −6 kcal/mol, while that for binding of biotin is +6 kcal/mol.

Two very different techniques have been used to determine the thermodynamics of interaction of BirA with biotin and bio-5′-AMP. An equilibrium dissociation constant for the BirA−biotin binding process has been determined by isothermal titration calorimetry at 25 °C. The excellent agreement between this value and that calculated from the kinetic parameters provides additional justification for the assumptions used in analysis of the kinetic data. Moreover, the reasonable agreement between the parameters obtained from van't Hoff analyses of the equilibrium parameters calculated from kinetic data and the heats of binding directly measured by calorimetry lend support to the kinetic techniques utilized and the assumptions made in analysis of the kinetic data obtained for binding of both ligands.

The heat capacities associated with binding of BirA to biotin and bio-5′-AMP were obtained from the measured temperature dependencies of the binding enthalpies. The enthalpic contribution to both binding processes increases with increasing temperature. The values of ΔC_p obtained are −160 cal/(K·mol) for biotin and −150 cal/(K·mol) for bio-5′-AMP. The magnitudes of both values are relatively small and are, within error, consistent with the results of van't Hoff analyses. The thermodynamic profiles for interaction of BirA with biotin and bio-5′-AMP have been calculated from the equilibrium constants derived from kinetic data and the results of isothermal titration calorimetric measurements (Figure 8). The heat capacity change for the binding reactions is assumed to be constant over the entire temperature range in this analysis. The Gibbs free energy for binding of BirA to the two ligand is also constant over the temperature range. While the enthalpy for binding of the two ligands is negative for both binding processes over the entire range, the entropic contribution to the biotin binding energetics is unfavorable and that for bio-5′-AMP binding is favorable. This enthalpy−entropy compensation observed in biotin binding results in the lower Gibbs free energy of binding of this ligand to BirA over the entire temperature range.

Relationship of Thermodynamic Parameters to Structure in the BirA System. Some high-resolution structural data are available for the BirA system. Structures of the aporepressor and the BirA−biotinyllysine complex have been determined by X-ray crystallography (Wilson et al., 1992). The protein is comprised of three structural domains. The central domain consists of a seven-stranded mixed β sheet surrounded by five α helices. A solvent-exposed face of the sheet contains the biotinyllysine and, by inference, the biotin and bio-5′-AMP binding site. Two disordered segments of the polypeptide chain including residues 116−124 and residues 212−223 are found in the region of the binding site. Residues 116-118 undergo a disorder to order transition upon binding biotinyllysine. We have obtained evidence from partial proteolysis for the occurrence of more extensive structural changes upon binding of biotin and bio-5′-AMP to the protein. Limited proteolysis indicates that the bond between residues 217 and 218 is sensitive to subtilisin-catalyzed cleavage (Xu et al., 1995). While saturation of the protein with biotinyllysine leads to no protection of this bond from subtilisin digestion (unpublished results), significant protection is observed when the protein is bound to biotin or bio-5′-AMP (Xu et al., 1995). Results of stopped-flow fluorescence measurement of association of BirA with biotin and bio-5′-AMP further support the occurrence of conformational changes in the protein upon binding of the ligands (Xu et al., 1995).

Since no structures of complexes of BirA with either biotin or bio-5′-AMP are available at this time, it is not yet possible to directly relate the thermodynamic parameters for binding to known structures of the free protein and ligands and the structures of the complexes. The structural basis of the binding energetics can, however, be calculated from the measured thermodynamic parameters (Gomez & Freire, 1995). The measured heat capacity changes and binding enthalpies at the specified temperatures are related to changes in solvent accessibility of both polar and apolar surface area by the following expressions (Murphy et al., 1992; Xie & Freire, 1994):

$$C_p(25\ ^\circ\mathrm{C}) = -0.26\Delta A_{\mathrm{polar}} + 0.45\Delta A_{\mathrm{apolar}} \quad (7)$$

$$\Delta H(60) = 31.4\Delta A_{\mathrm{polar}} - 8.44\Delta A_{\mathrm{apolar}} \quad (8)$$

where $\Delta A_{\mathrm{polar}}$ refers to the change in solvent-accessible polar surface area and $\Delta A_{\mathrm{apolar}}$ is the change in the solvent-accessible apolar surface area that occurs upon complex formation. $\Delta H(60)$ is the total binding enthalpy at 60 °C and is calculated from the measured binding enthalpies and the estimated heat capacity changes. Results of the calculations indicate that the burial of polar and apolar surface area contribute nearly equally to the energetics of the two binding reactions (Table 5). The magnitudes of the accessible surface area changes are significantly greater for biotin than for bio-5′-AMP. The combined binding free energies calculated from the kinetic data as well as the calorimetrically deter-

Table 5: Structural Parameterization of the Energetics of Binding of BirA to Biotin and Bio-5′-AMP

ligand	ΔG^a	ΔH^a	$\Delta A_{pol}{}^b$	ΔA_{np}	$-T\Delta S_{solv}{}^{a,c}$	$-T\Delta S_{rt}{}^{a,c}$	$-T\Delta S_{conf}{}^{a,c}$
biotin	−9.9	−15.6	−943	−902	−12.8	2.3	16.2
bio-5′-AMP	−13.9	−7.9	−640	−698	−12.0	2.3	3.6

[a] Energies given in kcal/mol and were calculated at 20 °C. [b] Calculated using eq 7. Units of area are Å^2. [c] Calculated using eq 8.

mined binding enthalpies and heat capacity changes can be used to further dissect the energetic contributions to the two binding reactions. The major contributions to the entropy change associated with a binding reaction arise from four sources which are related to the total entropy change by the following expression:

$$\Delta S = \Delta S_{solv} + \Delta S_{rt} + \Delta S_{ion} + \Delta S_{conf} \qquad (9)$$

ΔS is the total entropy change for the binding reaction, ΔS_{solv} is the entropy associated with solvent reorganization, ΔS_{rt} refers to the loss in rotational and translational entropy, ΔS_{ion} is the entropy change associated with ion or proton binding events that may be coupled to the binding of the protein and ligand, and ΔS_{conf} is the change in configurational entropy that occurs upon complex formation. On the basis of the measured small dependence of the binding enthalpy on buffer composition, we have assumed that the ionization entropy is equal to zero for both binding processes. The solvation entropy has been shown to be approximately equal to $\Delta C_p \ln(T/385.15)$, and we have used a rotational-translational entropy term of −8 cal/(K·mol) (Kauzmann, 1959; Murphy et al., 1993). Dissection of the binding energetics for the BirA−ligand interactions is shown in Table 5. The most striking result of these calculations is the significant difference in the conformational entropy term for the binding of the two ligands to BirA. Based on this difference, the magnitude of the structural change that occurs upon biotin binding is predicted to be much greater than that associated with adenylate binding. The results of partial proteolytic digestion of the unliganded and liganded forms of BirA indicate that binding of the adenylate to BirA results in greater protection of one region of the polypeptide chain from hydrolysis than does biotin binding, suggesting that bio-5′-AMP, not biotin, binding is linked to a greater disorder to order transition in the protein. One possible explanation for the discrepancy is that proteolytic digestion probes only a very limited region of the protein structure. It is possible that binding is coupled to changes in the conformation of other regions of the protein structure and these changes may be significantly different in binding of the two ligands. Alternatively, the expressions used to calculate the structural features of the two binding reactions require further refinement. In any case, the predictions of the calculations of the structural basis for the binding energetics will ultimately be tested as the high-resolution structures of the two liganded forms of BirA become available.

SUMMARY

The thermodynamics of association of BirA with biotin and bio-5′-AMP have been determined using a combination of kinetic and direct calorimetric measurements. Both reactions were found to be driven by large favorable enthalpies, and neither is coupled to significant proton uptake or release at pH 7.5. The entropic contribution to biotin binding is opposite in sign to that determined for bio-5′-AMP binding. This difference in the entropic contribution is the basis for the lower affinity of BirA for biotin. Results of structural parameterization of the two binding reactions indicates that burial of equivalent apolar and polar surface area occurs upon binding of the two ligands to BirA. The calculations indicate, moreover, that biotin binding is linked to a considerably greater loss in configurational entropy in the system than is binding of the adenylate. This loss in configurational entropy appears to be the major source of the unfavorable entropic contribution to biotin binding.

ACKNOWLEDGMENT

This work was performed in part at the Biocalorimetry Center, a Biomedical Research Technology Resource Center sponsored by the National Institutes of Health (RR04328), Johns Hopkins University, Department of Biology, Baltimore, Maryland. We thank Dr. Ernesto Freire for helpful comments on the manuscript.

REFERENCES

Abbott, J., & Beckett, D. (1993) *Biochemistry 32*, 9649−9656.
Barker, D. F., & Campbell, A. M. (1981a) *J. Mol. Biol. 146*, 451−467.
Barker, D. F., & Campbell, A. M. (1981b) *J. Mol. Biol. 146*, 469−492.
Cronan, J. E., Jr. (1989) *Cell 58*, 527−529.
Eisenberg, M. A., Prakash, O., & Hsiung, S.-C. (1982) *J. Biol. Chem. 257*, 15167−15173.
Gomez, J., & Friere, E. (1995) *J. Mol. Biol. 252*, 337−350.
Gomez, J. Hilser, V. J., Xie, D., & Freire, E. (1995) *Proteins: Struct., Funct., Genet. 22*, 404−412.
Kauzmann, W. (1959) *Advan. Protein. Chem. 14*, 1−63.
Jencks, W., P. (1987) *Catalysis in Chemistry and Enzymology*, pp 599−612, Dover Publications, Inc., New York.
Murphy, K. P., Bhakuni, V., Xie, D., & Freire, E. (1992) *J. Mol. Biol. 227*, 293−306.
Murphy, K. P., Xie, D., Garcia, K. C., Amzel, L. M., & Freire, E. (1993) *Proteins: Struct., Funct., Genet. 15*, 113−120.
Murphy, K. P., Xie, D., Thompson, K. S., Amzel, L. M., & Freire, E. (1994) *Proteins: Struct., Funct., Genet. 18*, 63−67.
Prakash, O., & Eisenberg, M. A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5592−5595.
Ross, P. D., & Subramanian, S. (1981) *Biochemistry 20*, 3096−3102.
Spolar, R. S., & Record, M. T., Jr. (1994) *Science 263*, 777−784.
Spolar, R. S., Ha, J.-H., & Record, M. T., Jr. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8382−8385.
Sturtevant, J. M. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2236−2240.
Taylor, John, R. (1982) *An Introduction to Error Analysis*, pp 40−80, University Science Books, Mill Valley, CA.
Wilson, K., Shewchuk, L. M., Otsuka, A. J., & Matthews, B. W. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 9257−9261.
Xie, D., & Freire, E. (1994) *Proteins: Struct., Funct., Genet. 19*, 291−301.
Xu, Y., & Beckett, D. (1994) *Biochemistry 33*, 7354−7360.
Xu, Y., Nenortas, E., & Beckett, D. (1995) *Biochemistry 34*, 16624−16631.

BI9600658