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Cooperative Binding of the *Escherichia coli* Repressor of Biotin Biosynthesis to the Biotin Operator Sequence†

Jennifer Abbott and Dorothy Beckett*

Department of Chemistry and Biochemistry, University of Maryland Baltimore County, Baltimore, Maryland 21228

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ABSTRACT: Regulation of biotin biosynthesis and retention in *Escherichia coli* depends on a complex set of coupled protein–protein, protein–nucleic acid, and protein–small molecule interactions. The complexity of the biotin system is analogous to that found in gene regulatory systems from other prokaryotes and from eukaryotes. Quantitative understanding of these systems requires thermodynamic studies of the individual contributing interactions. We have initiated such studies of the biotin regulatory interactions. The assembly states of the biotin operon repressor (BirA) and its complex with the allosteric effector, bio-5′-AMP, have been determined by analytical gel filtration chromatography. Both the apo- and holo-repressors are monomeric at protein concentrations several orders of magnitude higher than those required for DNA binding. Results of stoichiometric DNA binding measurements indicate that the BirA–biotin operator (bioO) complex consists of two holo-repressor monomers per operator site. Equilibrium binding of BirA to bioO has been measured using the quantitative DNase footprint technique. Analysis of the data indicates that the binding process is best described by a cooperative model. An upper limit for the cooperative free energy is estimated to be between –2.0 and –3.0 kcal/mol.

Regulation of transcription frequently depends on a variety of macromolecular interactions including those between protein and protein, protein and small molecules, and protein and DNA. There are, moreover, many regulatory systems in which these individual interactions are thermodynamically coupled. A quantitative understanding of transcriptional regulation, consequently, requires elucidation of the essential structural and thermodynamic features of the individual interactions *and* the mechanism of coupling of these interactions. Regulation of biotin biosynthesis and retention in *Escherichia coli* results from a complex set of coupled interactions of the three types indicated above (Cronan, 1989). Quantitative studies of the biotin system may therefore contribute to a general understanding of how individual macromolecular interactions work in concert to effect a transcriptional regulatory response.

Biotin biosynthesis in *E. coli* is regulated via a complex feedback inhibition mechanism (Cronan, 1989) that requires two protein molecules (BirA and BCCP), ATP, biotin, and the specific DNA sequence bioO. The 33.5-kDa protein BirA catalyzes covalent attachment of biotin to a lysine residue of the biotin carboxyl carrier protein (BCCP), a subunit of the predominant biotin-dependent enzyme in *E. coli*, the acetyl-CoA carboxylase. The small molecule substrates in the biotin ligation reaction are ATP and biotin with biotinyl 5′-adenylate (bio-5′-AMP) serving as an enzyme-bound intermediate (Prakash & Eisenberg, 1979). BirA also functions as the transcriptional repressor of biotin biosynthesis by binding to a 40 base pair interrupted palindrome sequence (bioO) to repress transcription initiation from the two divergent promoters, P_a and P_b, of the biotin biosynthetic operon (Otsuka & Abelson, 1978). The enzymatic intermediate, bio-5′-AMP, has the additional function of corepressor for sequence-specific binding of BirA to bioO (Eisenberg et al., 1982). In its capacity as corepressor, the adenylated form of biotin serves as a feedback inhibitor of its own synthesis. In conditions in which the metabolic requirement for biotin has not been satisfied, synthesis of the biotin biosynthetic enzymes is constitutive. Once the cellular requirement for biotin is fulfilled, synthesis of these enzymes is repressed. The switch from constitutive synthesis of biotin to repression thus involves a switch in BirA function from that of enzyme to sequence-specific DNA binding protein.

The complexity of the biotin regulatory system makes it a relevant model for many transcriptional regulatory systems. The individual macromolecular interactions that contribute to the biotin regulatory process include those between BirA and bio-5′-AMP, BirA and BCCP, and BirA and DNA. As indicated above, it is known that the first and third interactions are coupled. The existence of thermodynamic linkage between bio-5′-AMP and BCCP binding to BirA remains an open question. All of the biotin regulatory interactions are amenable to quantitative studies since both BCCP and BirA can be obtained in large quantities (Buoncristiani & Otsuka, 1988; Li & Cronan, 1992; Nenortas and Beckett, unpublished results). The recent determination of the three-dimensional structure of BirA will, moreover, allow correlation of thermodynamic measurements to structure (Wilson et al., 1992).

As initial steps in the quantitative study of the biotin regulatory system, we have characterized the assembly state of BirA and its complex with bio-5′-AMP. Both the apo- and holo-repressors are monomeric at concentrations well above those at which operator DNA binding occurs. We have also examined the mechanism of binding of the BirA–bio-5′-AMP complex to the biotin operator sequence. Sequence-specific binding of BirA to bioO occurs via cooperative binding of two BirA monomers to the two biotin operator half-sites. We propose that the lack of homologous protein–protein interactions between BirA monomers in the absence of DNA reflects an evolutionary constraint imposed by the heterologous protein–protein interaction with the BCCP. Furthermore,

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* Author to whom correspondence should be addressed.

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cooperative binding of two BirA monomers to bioO is advantageous for simultaneous repression of transcription from the two divergent promoters of the biotin operon.

MATERIALS AND METHODS

Plasmids and Strains. Plasmid pJMR1 (Otsuka and Junichi, unpublished results) contains a fusion of the birA gene to the tac promoter. The plasmid containing the biotin operator, pAOC3, has been described in Otsuka and Junichi (manuscript in preparation). pBioZ was constructed by inserting the 210 base pair *Eco*RI biotin operator fragment from pAOC3 into the *Eco*RI site of the plasmid pZ150 (Zagursky & Berman, 1984). All plasmids and proteins were purified from the *Escherichia coli* strain X90 [*ara* Δ(*lac-pro*) *nalA argE*am *rif thi*-1/F′ *lacI*^{Q} *lac*$^+$ *pro*$^+$] (Amman et al., 1983). Standard methods of microbiology were performed as described in Maniatis et al. (1982).

Enzymes and Chemicals. Restriction enzymes were obtained from Promega and BRL. Klenow fragment and DNA ligase were from BRL and Promega, respectively. Unlabeled deoxynucleoside triphosphates were from P. L. Biochemicals. α-^{32}P-labeled deoxynucleoside triphosphates used in end-labeling of DNAs were purchased from Amersham. Biotin, 5′-AMP, and ATP were from Sigma Chemicals. All chemicals used in preparation of buffers were reagent or analytical grade.

Protein Purification. BirA was overexpressed from plasmid pJMR1 in *E. coli* strain X90. Cells were grown in 2× YT media containing 100 μg/mL ampicillin; 75 mL of a late-log culture was used to inoculate 1 L of media. Once this culture had grown to OD_{600} = 0.8, IPTG was added to a final concentration of 100 μg/mL, and the culture was allowed to grow for an additional 5 h. The cells were harvested by centrifugation and resuspended in 2× the wet cell weight of lysis buffer (100 mM sodium phosphate, 100 mM NaCl, and 5% glycerol, pH 7.5). Cells were disrupted by sonication, and lysis was considered complete when the optical density at 600 nm had decreased by 90%. The lysate was diluted 5-fold in lysis buffer, and the resulting mixture was cleared by centrifugation at 9000 rpm for 45 min at 4 °C. Nucleic acids were precipitated by addition of 10% (v/v) poly(ethylenimine) (Sigma), pH 7.0, to a final concentration of 0.1% (v/v). The precipitate was pelleted by centrifugation at 10 000 rpm for 15 min. A saturated solution of ammonium sulfate was added to a final concentration of 60%, and the protein was allowed to precipitate at 4 °C overnight. The precipitate was pelleted at 10 000 rpm for 45 min at 4°C, and the resulting pellet was resuspended in 100 mM sodium phosphate, pH 6.5, 5% glycerol, and 1 mM DTT and dialyzed 3 times against the same buffer.

Chromatography. The ammonium sulfate precipitate obtained from a 10-L culture was chromatographed on a P11 cellulose phosphate (Whatman) column (5.5 × 35 cm) equilibrated in the same buffer as that used for dialysis of the sample above. Proteins were eluted in a linear NaCl gradient (0–0.8 M) in 100 mM sodium phosphate (pH 6.5)/5% glycerol, in which BirA elutes at approximately 0.6 M NaCl. Fractions containing BirA were pooled and dialyzed against 50 mM potassium phosphate, pH 6.5, 5% glycerol, and 1 mM DTT. The sample was chromatographed on hydroxylapatite–ULTROL (Sepracor) (2.5 × 35 cm) equilibrated in 50 mM potassium phosphate, pH 6.5, 5% glycerol, and 1 mM DTT. Proteins were eluted in a linear potassium phosphate gradient (50–500 mM) in which BirA eluted at a phosphate concentration of approximately 300 mM. Fractions containing BirA were pooled and dialyzed against 50 mM Tris-HCl, pH 7.4 at 4.0 °C, 100 mM KCl, 5% glycerol, and 0.1 mM DTT. This sample was chromatographed on S-Superose Fastflow (Pharmacia—LKB) (2.3 × 13 cm) equilibrated in the dialysis buffer and the protein was eluted using a linear KCl gradient (50 mM–1.0 M). BirA eluted at approximately 300 mM KCl. Samples containing BirA were pooled and dialyzed against 50 mM Tris, pH 7.5 at 4 °C, 200 mM KCl, 5% glycerol, and 0.1 mM DTT. The protein was stored in this buffer at −70°C. The total yield of BirA per gram of wet cell weight was 1.5 mg. The final protein sample was judged to be >95% pure by Coomassie staining of samples electrophoresed on an SDS–polyacrylamide gel. An extinction coefficient of BirA of 1.3 mg^{-1} mL was determined using the method of Gill and von Hippel (1989) and a modification of the procedure outlined in Pajot (1976). This extinction coefficient was utilized in determining protein concentration.

Chemical Synthesis of Bio-5′-AMP. Bio-5′-AMP was synthesized using the method outlined in Lane et al.(1965). The product was purified by ion-exchange chromatography on DEAE Sephadex A-25 resin (Sigma). The column (1.5 × 20 cm) was equilibrated in 0.02 M triethylammonium-bicarbonate (TEAB), pH 7.5 at 4 °C, and the sample was loaded in the same buffer and eluted in a linear gradient of TEAB (0.02–0.6 M). Fractions containing bio-5′-AMP were pooled, and the resulting sample was lyophilized. The product was desalted by reverse-phase chromatography on C-18 Sep-pack columns (Waters). The final sample was lyophilized and stored desiccated at −20 °C.

Fluorescence Titrations. Fluorescence measurements were made using an SLM 48000 spectrofluorometer. Measurements were performed in buffer containing 10 mM Tris-HCl, 200 mM KCl, 2.5 mM $MgCl_2$, and 1 mM $CaCl_2$, pH 7.50 ± 0.02 at 20 °C. The sample temperature was maintained at 20.0 ± 0.1 °C with a circulating water bath. The excitation wavelength was 295 nm, and emission was monitored from 310 to 450 nm. Excitation and emission slit widths were set at 4 nm. All measurements were made in the ratio mode using rhodamineB as a quantum counter. Titrations were performed by sequential addition of small volumes of concentrated bio-5′-AMP to a solution containing BirA. The mixtures were allowed to equilibrate for at least 5 min prior to measurement of the spectra. Spectra were corrected for the contribution of buffer and volume changes. The fluorescence intensities utilized in analysis of titration data were obtained by integration of the background- corrected fluorescence spectra. No correction was made for the inner filter effect because the extinction coefficient for the adenosine moiety of bio-5′-AMP is zero at 295 nm.

Analytical Gel Filtration Chromatography. Analytical gel filtration chromatography (Ackers, 1975) of BirA was performed on Sephadex G-100 resin (Pharmacia). The column dimensions were 1.2 × 30 cm. Samples were equilibrated and chromatographed in buffer containing 10 mM Tris-HCl, pH 7.5 at 20 °C, 200 mM KCl, and 2.5 mM $MgCl_2$. The column flow rate was maintained at approximately 0.25 mL/min using a Pharmacia Perpex peristaltic pump. Eluted zones were detected using an ISCO Model 1840 absorbance monitor. Small zones of bovine serum albumin, ovalbumin, chymotrypsinogen, and ribonuclease A (Pharmacia) were run as calibration standards. Both large (V_T = 25 mL) and small (V_T = 0.5 mL) zones of BirA were chromatographed. Protein zones run in the presence of bio-5′-AMP were chromatographed within a larger zone of the small molecule. Centroids for the leading and trailing edges of large zones were determined by planimetry. The partition

coefficient, σ, of a protein on the column was calculated from the elution volume of the zone:

$$\sigma = (V_e - V_o)/V_i \quad (1)$$

where V_e is the elution volume of the zone, V_o is the void volume of the column, and V_i is the included volume. V_o is determined from the elution position of a small zone of Blue Dextran while $V_i + V_o$ was determined by running a small zone of the nucleoside adenosine. Partition coefficients were used to estimate the apparent Stokes radii of BirA samples relative to proteins of known Stokes radii using the expression (Ackers et al., 1975):

$$a = a_0 + b_0 \, \mathrm{erfc}^{-1} \sigma \quad (2)$$

where a is the molecular radius, σ is the partition coefficient, and erfc^{-1} is the inverse error function complement. The slope, b_0, and intercept, a_0, are calibration constants for a given gel.

Plasmid and Biotin Operator Fragment Preparation. Fragments used for DNA binding measurements were obtained from either plasmid pAOC3 or plasmid pBioZ. The plasmids were prepared from *E. coli* strain X90 according to the method of Birnboim and Doly (1979) and were finally purified by CsCl density gradient centrifugation (Maniatis et al., 1982). The 210 base pair fragment used for stoichiometry measurements was obtained by cleavage of the plasmid pAOC3 with *Eco*RI; 1000 base pair fragments used in DNase footprinting experiments were obtained by digestion of the plasmid pBioZ with *Hin*dIII and *Pst*I. Reaction products were separated on 1% agarose gels, and operator fragments were electroeluted from the agarose according to the method outlined in Maniatis et al. (1982). DNA fragments were labeled with ^{32}P as described in Brenowitz et al. (1986).

DNA Binding Measurements. (A) Nitrocellulose Filter Binding. Nitrocellulose filter binding measurements were performed as described in Senear et al. (1986). The buffer used for equilibrium binding measurements contained 10 mM Tris-HCl, pH 7.5 at 20 °C, 200 mM KCl, 2.5 mM $MgCl_2$, 2 μg/mL sonicated calf thymus DNA, 100 μM biotin, and 1 mM ATP. At these concentrations of the two substrates, biotin and ATP, under these buffer conditions, BirA is saturated with bio-5′-AMP (Xu and Beckett, unpublished results). Moreover, results of biotin operator binding measurements performed either in the presence of a large excess of chemically synthesized bio-5′-AMP (100 μM) or in the presence of the two substrates biotin and ATP are identical. Stoichiometry measurements were performed using the same buffer with the exception that the KCl concentration was 50 mM rather than 200 mM. For equilibrium binding measurements, the DNA concentration is insignificant relative to the apparent midpoint of the tirations so that the assumption that $[\mathrm{BirA}]_{tot} = [\mathrm{BirA}]_{free}$ is valid.

DNase Footprint Titrations. DNase footprint titrations were performed as described in Brenowitz et al. (1986). The buffer used for these measurements was identical to that used for equilibrium nitrocellulose filter binding measurements. The products were separated on 10% denaturing polyacrylamide gels, which were then exposed to preflashed X-ray film (Kodak XAR5). Densitometric analyses of autoradiograms were performed using a Molecular Dynamics personal laser densitometer. Due to scattering of the laser beam as it passes through the X-ray film, optical densities obtained directly by scanning deviate positively from the actual values. The deviations were measured over a broad OD range by scanning a precalibrated stepwedge (Kodak). A linear relationship between the magnitude of the deviation and the calibrated optical density value reported by the manufacturer of the stepwedge was found to exist over the optical density range from 0 to 2.4 optical density units (ODU), a range that exceeds the linear range of the response of X-ray film (0.2–1.8 ODU). Data were corrected using this linear relationship.

Data Analysis. Data were analyzed by nonlinear least-squares parameter estimation methods using the program Nonlin (Straume *et al.*, 1991). All calculations were performed on a Silicon Graphics 4D-220GTXB computer. Models used for analysis of the footprinting data are described in the text.

RESULTS

Purification of BirA. The method for large-scale purification of BirA described in this work differs considerably from the previously published method (Buoncristiani et al., 1988). The timing of the cell growth and the length of IPTG induction of BirA synthesis were changed. Disruption of the cells was performed by sonication alone instead of lysozyme treatment followed by sonication. The lysate was treated with poly(ethylenimine) to remove nucleic acids and then subjected to ammonium sulfate precipitation prior to chromatography. As in the previous purification, the protein was subjected to three chromatography steps. The resins used in this work and the details of the chromatography differ considerably from the chromatography previously described.

The final yield of BirA obtained using the new purification method is greater than that reported using the previously published procedure. The fractional activity of the preparation in binding bio-5′-AMP was determined by a fluorescence titration method. Fluorescence spectra of BirA alone and in its complex with bio-5′-AMP are shown in Figure 1a. Saturation of BirA with bio-5′-AMP results in quenching of 41% of the intrinsic protein fluorescence signal. This fluorescence change was used to estimate the activity of the protein preparation by performing a titration of BirA with bio-5′-AMP under stoichiometric conditions (Figure 1b). Analysis of the data using a simple breakpoint titration model indicates a fractional activity of 0.94 ± 0.04. Measurements of the activities of birA preparations in biotin binding (Pattaseril and Beckett, unpublished results), catalysis of bio-5′-AMP synthesis (Xu and Beckett, unpublished results), and bioO binding (see below) have also been performed, and all indicate an activity close to 100%. Furthermore, the protein can be stored at −70 °C for as long as 2 years with no loss of activity.

BirA Is a Monomer. The assembly state of BirA was determined by analytical gel filtration chromatography on Sephadex G-100 resin. Chromatography was performed in buffer identical to that used for DNA binding measurements with the exception that carrier calf thymus DNA and BSA were omitted. Both small and large protein zones were chromatographed over a concentration range from 0.2 to 2 μM. Large zones of BirA exhibited symmetrical shapes (data not shown), and similar elution volumes were determined for BirA loaded as either a large or a small zone. These results suggest that BirA exists as a single non-dissociating species in solution. The Stokes radii of BirA samples chromatographed over a range of conditions are similar (Figure 2). The data were used to calculate the apparent molecular weight of BirA using the expression:

$$\sigma = -A \log M_r + B \quad (3)$$

where σ is the partition coefficient, M_r is the molecular weight, and A and B are calibration constants for the column. The Stokes radii and calculated molecular weights determined for

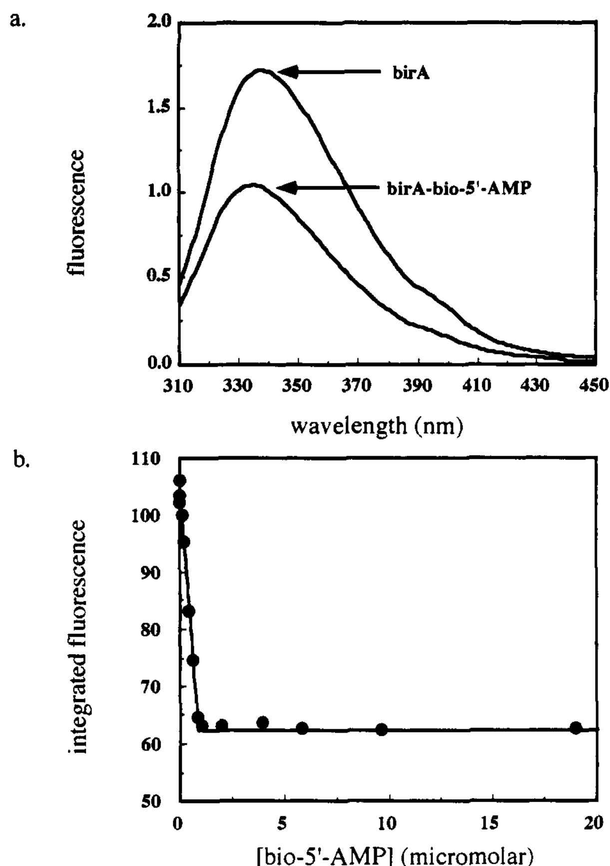

FIGURE 1: (a) Intrinsic protein fluorescence spectra of free BirA and BirA complexed to bio-5′-AMP. The units of fluorescence are arbitrary. (b) Stoichiometric curve for titration of BirA with bio-5′-AMP. The solid line represents the best-fit curve obtained from nonlinear least-squares analysis of the data using a simple relation for stoichiometric binding of the ligand to BirA. [BirA] = 0.96 μM.

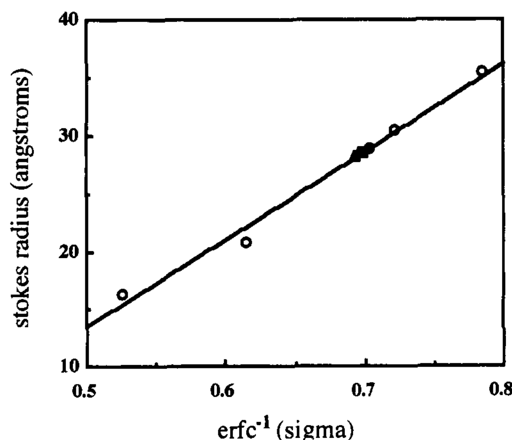

FIGURE 2: Analytical gel filtration chromatography of BirA (■) and the BirA–bio-5′-AMP complex [(▲) 2 μM effector; (●) 8 μM effector] on Sephadex G-100 resin. Chromatography was carried out in 10 mM Tris-HCl, pH 7.5, 200 mM KCl, 2.5 mM $MgCl_2$, and 1 mM $CaCl_2$ at 20 °C. The open circles represent protein calibration standards (Materials and Methods), and the solid line represent the best fit of the data to eq 2 (Materials and Methods).

unliganded BirA are shown in Table I. The calculated molecular weights are somewhat higher than the expected value of 33 500 based on the amino acid sequence. This is likely due to the fact that the protein is asymmetric with an axial ratio of approximately 2 (Wilson et al., 1992). Identical results were obtained regardless of whether the protein was chromatographed as a large or a small zone. All of the data

Table I: Results of Analytical Gel Chromatography Measurements on Sephadex G-100[a]

[BirA] (μM)	[bio-5′-AMP] (μM)[b]	Stokes radius[c] (Å)	MW(app)[d]
1.44	none	28.2 (0.2)	36117
1.28	2	28.6 (0.4)	37214
1.58	8	28.9 (0.2)	38346
42 (small zone)		28.7 (0.4)	

[a] Zones of BirA (large zones, 25 mL; small zone, 0.5 mL) were chromatographed at the indicated concentrations in the absence and presence of chemically synthesized bio-5′-AMP. [b] Zones run in the presence of the effector were chromatographed within a larger zone of chemically synthesized bio-5′-AMP. [c] Stokes radii (with absolute uncertainties) were determined relative to standards (Figure 1) according to the method of Ackers (1967). [d] The molecular weights were calculated using eq 3 (see Results).

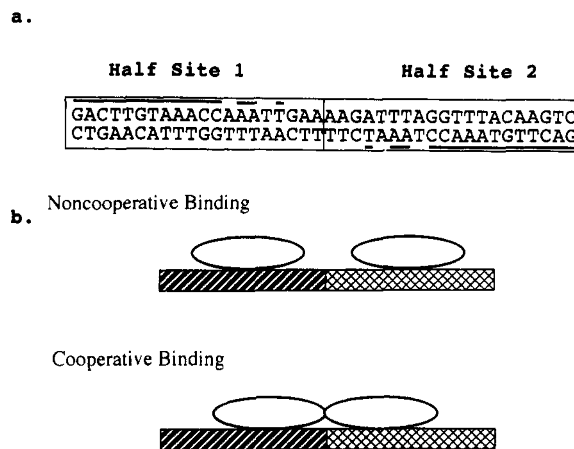

FIGURE 3: (a) Biotin operator sequence (Otsuka & Abelson, 1978). Underlined segments represent base pairs that are identical in the two half-operator sequences. The half-sites are arbitrarily identified as 1 and 2. (b) Noncooperative and cooperative models for the interaction of BirA with half-sites 1 and 2 of bioO. The direct contact shown between the two monomers in the cooperative model indicates the thermodynamic interaction between the simultaneously bound protomers. The structural basis for this interaction is discussed in the text.

are consistent with the conclusion that BirA is monomeric in solution.

The effect of the small ligand bio-5′-AMP on the assembly state of BirA was also examined. Large zones of BirA were chromatographed in the presence of either a stoichiometric amount or a molar excess of chemically synthesized bio-5′-AMP (Figure 1). Independent binding measurements in identical buffer conditions indicate an equilibrium dissociation constant for the BirA–bio-5′-AMP interaction in the subnanomolar range of concentration (Xu and Beckett, unpublished results). Bio-5′-AMP is, thus, stoichiometrically bound to BirA in the conditions used for these chromatography experiments. The partition coefficient of the protein–ligand complex was used to calculate its apparent molecular weight (Table I). These results indicate that the protein is also monomeric when bound to bio-5′-AMP. The concentration of BirA in the samples subjected to gel filtration chromatography is approximately1000-fold higher than the concentrations required for biotin operator binding (see below).

Two BirA Molecules Bind to the Biotin Operator Sequence. BirA binds sequence-specifically to the 40 base pair interrupted palidromic sequence, bioO (Figure 3a). The stoichiometry of the BirA–biotin operator complex was measured by the nitrocellulose filter binding technique under stoichiometric conditions. These conditions were determined by measuring the binding of BirA to the operator under conditions of protein

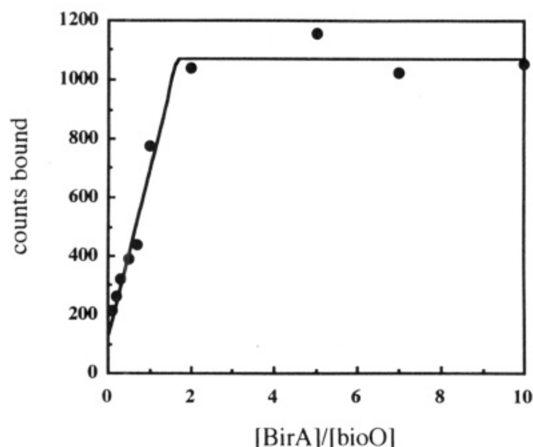

FIGURE 4: Binding curve for BirA binding to bioO under stoichiometric conditions. Buffer conditions are indicated under Materials and Methods. The solid line represents the best fit of the data to a breakpoint curve.

excess. The BirA concentration required for half-maximal saturation of the biotin operator in these buffer conditions is 0.5 nM. Stoichiometry measurements were performed at a total DNA operator concentration of 10 nM, and the results are shown in Figure 4. The data were fit to a simple breakpoint titration to obtain a stoichiometry of the complex of 1.7 + 0.3. The results are consistent with a stoichiometry of 2 BirA monomers per biotin operator site. The stoichiometry determination is based on the assumption that the protein is 100% active. An alternate, but unlikely in light of the results of the bio-5′-AMP binding titration shown above, interpretation of the stoichiometry data is that the protein is approximately 60% active and only one monomer binds to bioO.

BirA Monomers Bind Cooperatively to BioO. Binding of two BirA monomers to the two half-sites of the biotin operator can occur by either a cooperative or a noncooperative mechanism (Figure 3b). In the case of noncooperative binding, the total Gibbs free energy for the binding process is simply the sum of the intrinsic free energies, ΔG_1 and ΔG_2, for binding of a BirA monomer to half-sites 1 and 2, respectively. The intrinsic free energy refers to the free energy of binding to a half-site in the absence of binding to the other half-site. If the binding is cooperative, an additional cooperative or excess free energy term, ΔG_{12}, associated with simultaneous binding of the two monomers to the two half-site contributes to the stability of the BirA–bioO complex. Experimental determination of the correct binding model requires use of an individual site binding method that allows independent observation of the occupancy of each of the bioO half-sites at any BirA concentration. The DNase footprint titration technique was used for this determination. The individual site binding equations for the two half-sites are

$$Y_1 = \frac{k_1[\mathrm{P}] + k_1 k_2 k_{12}[\mathrm{P}]^2}{1 + (k_1 + k_2)[\mathrm{P}] + k_1 k_2 k_{12}[\mathrm{P}]^2} \quad (4a)$$

and

$$Y_2 = \frac{k_2[\mathrm{P}] + k_1 k_2 k_{12}[\mathrm{P}]^2}{1 + (k_1 + k_2)[\mathrm{P}] + k_1 k_2 k_{12}[\mathrm{P}]^2} \quad (4b)$$

where Y_1 and Y_2 represent the fractional saturation of site 1 and site 2, respectively, [P] is the protein concentration, and k_1 and k_2 are the microscopic or intrinsic equilibrium constants for binding of a BirA monomer to sites 1 and 2, respectively. k_{12} is the equilibrium constant for the cooperative interaction of the two monomers. The Gibbs free energies are obtained from the relation $\Delta G = -RT \ln k$.If the binding is noncooperative, the ΔG_{12} is zero.

A footprint titration of the biotin operator is shown in Figure 5. The pattern of cleavage of the operator is striking in terms of the resistance of the central region of the sequence to DNase cleavage either in the absence or in the presence of BirA. The sequence of this central region is A_4 (Figure 3), and such homopolymeric sequences have previously been shown to be

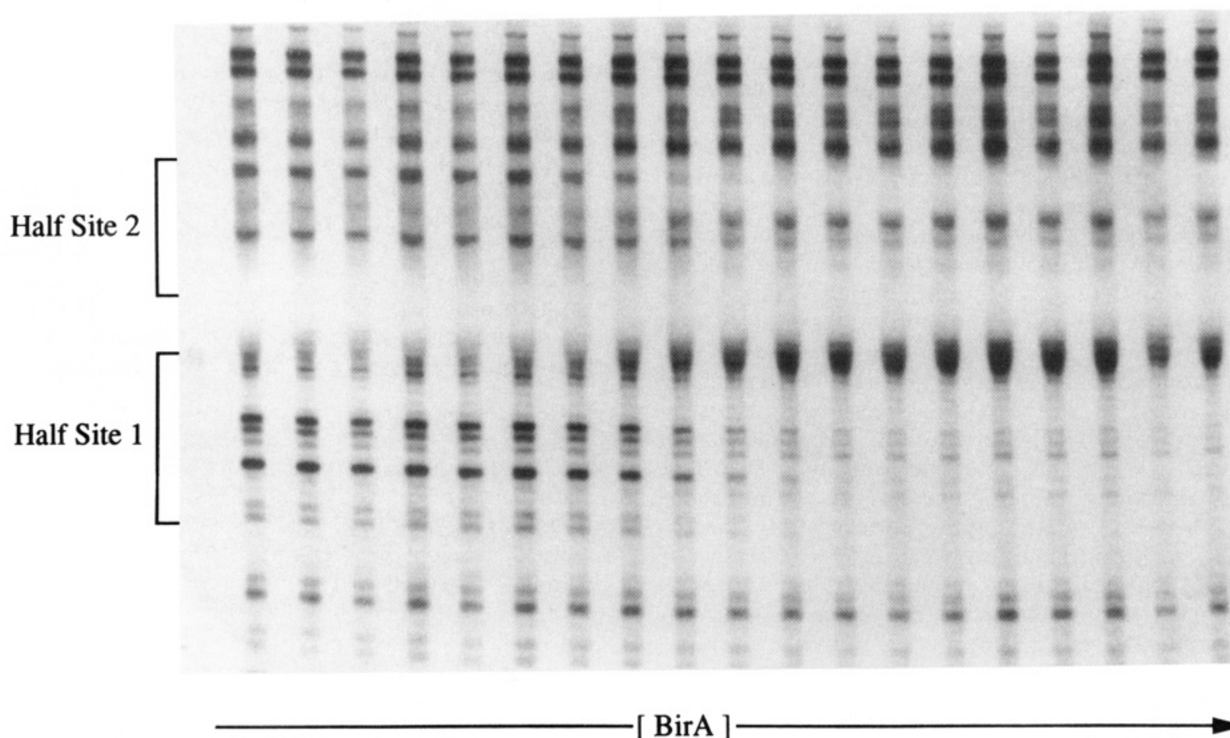

FIGURE 5: DNase footprint titration of the biotin operator region. The bands corresponding to the individual half-sites are labeled as half site 1 and half site 2. The BirA concentrations are indicated in Figure 6.

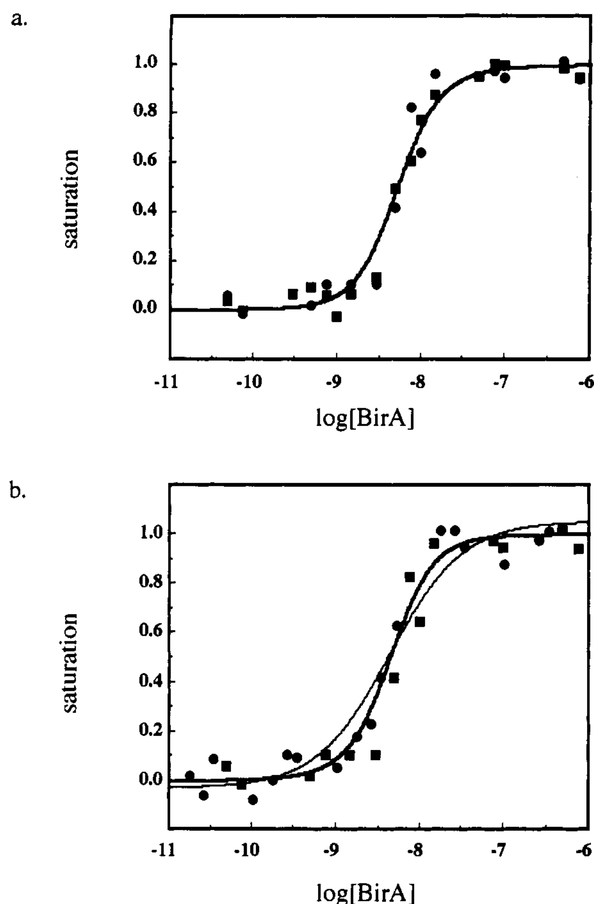

FIGURE 6: (a) Individual site binding curves for BirA binding the two half-sites of bioO. Half-operators 1 (●) and 2 (■) are indicated in Figure 2. The solid curves represent the best-fit values of ΔG_1 and ΔG_2 assuming a ΔG_{12} value of –2 kcal/mol. (b) Individual site binding curves for binding of BirA to half-site 2 of bioO. The thin solid line represents the best fit of the data assuming ΔG_{12} = 0 kcal/mol while the thick solid line represent the best fit of the data assuming ΔG_{12} = –2 kcal/mol. The two types of symbols represent data obtained from two separate experiments.

refractory to DNase I cleavage (Drew & Travers, 1984). The footprint of the site indicates that for bands that do change in intensity the change is, in most cases, a decrease. Two exceptions are a band in half-site 2 and the region adjacent to the center of the site which both increase in intensity as the occupancy of the site increases.

Isotherms for the individual half-sites were obtained by integrating the optical densities of a subset of the bands in each half-site over the entire titration. Results of this analysis are shown in Figure 6a. The two half-sites titrate at identical protein concentrations, and the binding transition for each half-site occurs over approximately 1 log unit of BirA monomer concentration. The steepness of the transitions is consistent with a cooperative binding mechanism. This limited set of data does not allow unequivocal determination of all three binding parameters, ΔG_1, ΔG_2, and ΔG_{12}, for the interaction of BirA with the operator region. Noncooperative binding can, however, be distinguished from cooperative binding by nonlinear least-squares fitting techniques. This was done by estimating the intrinsic Gibbs free energies for the monomer–half-site interactions assuming a range of fixed cooperative free energies from 0 to –3 kcal/mol. Results of the analyses for several assumed values of the cooperative free energy are shown in Figure 6b and in Table II. Comparison of the binding data for site 2 with results of analysis based on the assumption of noncooperative binding indicates that there is a systematic

Table II: Resolved Gibbs Free Energies of the Interaction of BirA with the Two Half-Sites of the Biotin Operator (BioO)[a]

ΔG_{12}[b,c]	ΔG_1	ΔG_2	σ[d]
0	–11.2 (0.3)	–11.0 (0.3)	0.086
–1.0	–10.8 (0.3)	–10.5 (0.3)	0.064
–2.0	–10.4 (0.4)	–9.9 (0.4)	0.056
–3.0	–10.1 (0.6)	–9.2 (0.6)	0.054

[a] ΔG_1 and ΔG_2 are the free energies for binding of a BirA monomer to BioO half-sites 1 and 2 (Figure 3), respectively. ΔG_{12} is the interaction energy for simultaneous binding of two monomers to the half-sites. [b] The binding free energies were estimated from analysis of titration data obtained from footprints of the wild-type operator. ΔG_{12} values were fixed at the values shown, and estimates of the two intrinsic Gibbs free energy terms for binding of the BirA monomer to each operator half-site were made. [c] Standard Gibbs free energies in kilocalories per mole (with 65% confidence limits). [d] Square root of the variance of the fitted curves.

Table III: Resolved Cooperative Free Energies for the Simultaneous Binding of Two BirA Monomers to the Two Half-Sites of the Biotin Operator (BioO)[a]

ΔG_{12}[b,c]	ΔG_1	ΔG_2	σ[d]
–0.4 (0.3)	–11.0	–11.0	0.077
–1.3 (0.2)	–10.5	–10.5	0.061
–2.3 (0.2)	–10.0	–10.0	0.055
–3.3 (0.2)	–9.5	–9.5	0.054

[a] ΔG_1 and ΔG_2 are the free energies for binding of a BirA monomer to bioO half-sites 1 and 2 (Figure 2), respectively. ΔG_{12} is the cooperative free energy term. [b] ΔG_1 and ΔG_2 were fixed at the values shown, and estimates of the cooperative Gibbs free energy for simultaneous binding of the two BirA monomers to the two operator half-sites were made. [c] Standard Gibbs free energies in kilocalories per mole (with 65% confidence limits). [d] Square root of the variance of the fitted curves.

deviation of the data from a noncooperative binding model. A better agreement of the data with the best-fit curve is observed for a cooperative model. The improvement of the fit to a cooperative model is also indicated by the values of the square root of the variance, 0.056 and 0.077, for cooperative and noncooperative models, respectively. Models that included a negative cooperative free energy term consistently yielded better values of the square root of the variance than models that assumed a value of zero or greater for ΔG_{12}. The data were also analyzed for the cooperative free energy term assuming fixed equivalent values for the ΔG_1 and ΔG_2 terms (Table III). Results of these analyses indicate that the goodness of fit, as judged by the value of the square root of the variance, is significantly improved for all fixed values of the two intrinsic free energies that result in best-fit values of the ΔG_{12} term less than –2.0 kcal/mol. The lack of change in the confidence intervals and the square root of the variance as the two intrinsic free energy terms are fixed at smaller negative values indicates that an upper limit of –2.0 to –3.0 kcal/mol can be placed on the cooperative free energy term.

DISCUSSION

The Assembly State of BirA. BirA, both in its free form and when complexed to bio-5′-AMP, is a monomer in solution. Eisenberg *et al.* (1982) have previously determined a native molecular weight for BirA of 40 000 using gel filtration chromatography, consistent with the results reported in this work. In the previous work, higher molecular weight species that exhibited biotin binding activity were also observed. These data were interpreted as indicating the exisitence of higher order oligomers of BirA. We have obtained no evidence for the existence of any oligomeric species of BirA in the absence of DNA. The observation that BirA is monomeric is consistent with the monomeric structure observed in the X-ray crys-

tallographically determined three-dimensional structure of BirA (Wilson et al., 1992). Structures obtained thus far are of the apo-repressor and the complex of BirA with biocytin (biotinoyllysine). The structure of the BirA–corepressor complex has yet to be determined. Bio-5′-AMP is a positive effector for binding of BirA to BioO, and a potential mechanism of corepressor action involves ligand-induced dimerization of BirA. This possibility is supported by the 2:1 stoichiometry of the BirA–bioO complex and the similarity of the BirA DNA binding region to that of other helix–turn–helix DNA binding proteins, the majority of which bind to their dyad symmetric sites as preformed oligomers (Brennan & Matthews, 1989). Results of analytical gel filtration chromatography measurements presented in this work indicate, however, that the complex of BirA with the adenylate is monomeric at concentrations well above those at which site-specific DNA binding is observed.

Cooperative Binding of Two BirA Monomers to BioO. The mechanism of binding of BirA to the biotin operator involves association of the two monomers with the two half-sites of the operator region. This stoichiometry is in agreement with that reported by Lin et al. (1992). The stoichiometric bioO and bio-5′-AMP binding data also indicates that the BirA preparations used in this work are nearly 100% active in DNA binding. All other measures of BirA activity including biotin binding (Pattaseril and Beckett, unpublished observations) and active-site titrations (Xu and Beckett, unpublished observations) are consistent with this result. Analysis of the DNase footprint titration data presented in this work indicates that the two BirA monomers bind cooperatively to the two bioO half-sites. Although the data are insufficient to determine the exact value of the cooperative free energy, the results clearly indicate that the data are best described by a cooperative model. We estimate that, in the buffer conditions employed, an upper limit for the Gibbs free energy for the cooperative interaction is −2.0 to −3.0 kcal/mol. The structural basis of this cooperativity is not known at this time. Results of genetic studies of BirA mutants that are defective in repression indicate the existence of two complementation groups (Barker & Campbell, 1980). One interpretation of this complementation is that it reflects dimerization of the protein. If such dimerization does occur, it must be a DNA-induced process since the protein and its complex with the corepressor are clearly monomeric at concentrations well above those required for DNA binding. The cooperativity observed in binding of the two monomers to the two bioO half-sites indicates a thermodynamic interaction between the two monomers. This interaction may, at the structural level, involve formation of a protein–protein interface.

The ability of the DNA to form a nonstandard structure may also play a role in the cooperative binding of BirA to BioO. The biotin operator is 40 base pairs in length and consists of two 18 base pair sequences nearly identical in sequence connected by a 4 base pair (dA)·(dT) tract. Such homopolymer tracts have been shown to form unusual, non-B-like structures (Nelson et al., 1987). The lack of DNase cleavage in this region of the operator suggests a structure that differs from standard B-form. Cooperative binding of BirA to the operator sequence may depend upon a unique structure of the operator sequence that allows optimal interaction of the monomers as they bind to DNA. Experiments designed to test this possibility are now in progress.

The helix–turn–helix DNA binding domain of BirA is structurally similar to that of a large number of prokaryotic repressors for which structures are available. Like these other proteins, BirA binds to a dyad symmetric operator site. In general, this class of proteins bind as preformed oligomers to their respective operators. One exception is the *E. coli* lexA repressor. The equilibrium dimerization constant for lexA is in the micromolar range of concentration while the protein concentration required to obtain half-maximal saturation of the recA operator is in the nanomolar range (Kim & Little, 1992). Results of DNase footprinting experiments indicate that lexA, like BirA, binds to a dyad symmetric operator site cooperatively and the cooperativity has been interpreted to indicate a DNA-induced dimerization process. Unlike lexA, no dimerization of BirA is detected at protein concentrations 2–3 orders of magnitude higher than the concentration required for operator binding.

Relationship of the DNA Binding Mechanism to the Biotin Ligase Function of BirA. BirA is a multifunctional protein that catalyzes the synthesis of biotinoyl-5′-AMP from the substrates biotin and ATP, catalyzes the transfer of biotin from the adenylate to a lysine residue of the biotin carboxyl carrier protein (BCCP) subunit of the acetyl-CoA carboxylase, and binds sequence-specifically to the biotin operator to repress transcription of the biotin biosynthetic genes. The lack of dimerization of BirA in the absence of DNA may be related to the multiple functions in which the protein participates. In particular, it is not known whether the BCCP interacts with a monomer or a dimer of BirA. The purified biotin ligases from *P. shermanii* (Shenoy, 1988), *Bacillus stearothermophilus* (Cazzulo et al., 1971), and *Saccharomyces cerevisiae* (Sundaram et al., 1971) are all monomeric. The protein substrate of the *P. shermanii* ligase is the 1.3S subunit of the methylmalonyl-CoA-carboxyltransferase, and it has been shown that BirA catalyzes transfer of biotin to the 1.3S subunit, as well as to a number of other heterologous biotin acceptor proteins (Cronan, 1989). The observation that BirA and the biotin ligases from a number of other organisms are monomers suggests that this is the oligomeric state that is relevant to the biotin-transfer reaction. The monomeric state of BirA may, therefore, reflect a constraint on the molecule imposed by this heterologous protein–protein interaction with the BCCP. Several lines of evidence suggest that control of the functional switch of BirA from its biotin ligase to its DNA binding function reflects competition between the apoBCCP and the biotin operator for binding to the BirA–bio-5′-AMP complex. The switch occurs only once the cellular pool of apoBCCP has been converted to the holo form, and overproduction of the 1.3S biotin acceptor protein results in derepression of the biotin operon (Cronan, 1988). One level at which the BCCP–BirA interaction may compete with the BirA–bioO interaction is by preventing dimerization of BirA monomers. The BCCP has recently been obtained in large quantities in our laboratory. Direct biophysical studies of this heterologous protein–protein interaction will provide information about the relationship of the assembly state of BirA to its enzymatic function.

Cooperative Binding of BirA and Coordinate Regulation of Transcription Initiation from the Two Biotin Operon Promoters. The cooperative nature of the BirA–bioO interaction may be related to specific structural features of the biotin operon control region. The biotin biosynthetic operon contains the genes that code for five of the six enzymes required for biotin biosynthesis (Eisenberg, 1984). Transcription of the five genes is initiated from two divergent promoters, P_a and P_b (Figure 7), which overlap both half-operators of bioO (Otsuka & Abelson, 1978). Coordinate regulation of all five genes in the operon in response to biotin concentration requires simultaneous repression of transcription initiation from both

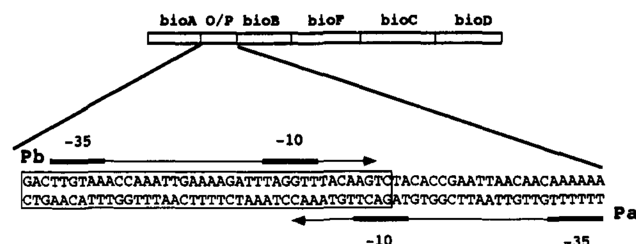

FIGURE 7: Schematic representation of the biotin biosynthetic operon and the detailed sequence of the biotin operon transcriptional control region. bioA through bioF are the genes that code for the biotin biosynthetic enzymes, O/P is the transcriptional control region for the operon, and Pa and Pb are the promoters for leftward and rightward transcription, respectively. The boxed sequence represents the biotin operator sequence.

promoters. Cooperative binding of the two BirA monomers to the two bioO half-sites may have advantages over noncooperative binding for such coordinate repression. Noncooperative binding would allow, at certain BirA concentrations, the existence of operators occupied by only one monomer. In such singly liganded species, the activity of one promoter may be repressed while transcription from the other is constitutive. Cooperative binding minimizes the contribution of such singly liganded species to the species population, thereby ensuring that coordinate repression of all five genes in the biotin operon is obtained.

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