Evidence for Interdomain Interaction in the *Escherichia coli* Repressor of Biotin Biosynthesis from Studies of an N-Terminal Domain Deletion Mutant†

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ABSTRACT: The *Escherichia coli* repressor of biotin biosynthesis (BirA) is an allosteric site-specific DNA binding protein. The protein is composed of three structural domains. Contact with the biotin operator (bioO) in the transcriptional repression complex is made by the N-terminal domain which contains a helix−turn−helix structural module. The central domain is required for the catalytic functions of BirA including synthesis of biotinyl-5′-AMP from substrates ATP and transfer of biotin from the adenylate to a lysine residue of the biotin carboxyl carrier protein (BCCP) of acetyl CoA carboxylase. The adenylate serves not only as the activated intermediate in the biotin transfer reaction but also as the positive allosteric effector for site-specific DNA binding. Little interaction between the N-terminal and central domains is observed in the apo-repressor structure (Wilson *et al.*, 1992). In this work, we have engineered an N-terminal deletion mutant of BirA, BirA65−321. Biochemical analysis of the purified truncated repressor indicates that, as expected, it does not bind to biotin operator DNA. BirA65−321 is, moreover, identical to intact BirA in catalysis of synthesis of bio-5′-AMP and in transfer of biotin from the adenylate to BCCP. Deletion of the DNA binding domain severely compromises the ability of BirA to bind to biotin or bio-5′-AMP. The affinity of BirA65−321 for biotin is decreased 100-fold while that for bio-5′-AMP is decreased 1000-fold, relative to intact BirA. The significant functional role of the DNA binding domain in tight binding of the two ligands to the central domain may be indicative of formation of extensive interdomain contacts in the holorepressor structure.

Site-specific DNA binding of a large number of transcriptional regulatory proteins is modulated by binding of small molecules. This modulation of activity is critical for enabling an organism to respond, at the level of gene expression, to changes in its environment. A combination of biochemical and genetic evidence, as well as high-resolution structural data, indicates that many of the regulatory proteins that respond to effector binding are modular in structure. DNA and effector binding functions are found in separate globular domains in several of these proteins including the *Escherichia coli* lac repressor (Platt *et al.*, 1973) and the cAMP receptor protein (CRP) (Eilen *et al.*, 1978; Aiba & Krakow, 1981; McKay *et al.*, 1982). Since the affinities of these proteins for their target sites on DNA are modulated by binding of small molecule effectors, interaction between the structural domains within these proteins is central to their function. The thermodynamic and structural details of the interdomain communication have not been determined.

The repressor of biotin biosynthesis, BirA, binds to the biotin operator, bioO, to repress transcription of the biotin biosynthetic operon (Prakash & Eisenberg, 1979; Barker & Campbell, 1981b; Otsuka & Abelson, 1978; Cronan, 1989). The affinity of BirA for the operator sequence is modulated by binding of the adenylated form of biotin, bio-5′-AMP (Prakash & Eisenberg, 1979). Unlike the majority of transcriptional regulatory proteins, BirA is also an enzyme and belongs to a class of proteins termed the biotin holoenzyme synthetases or biotin−protein ligases (Barker & Campbell, 1981a,b; Eisenberg *et al.*, 1982). These enzymes catalyze covalent ligation of biotin to the biotin-dependent carboxylases. The protein substrate for BirA is the biotin carboxyl carrier protein, BCCP, subunit of the acetyl-CoA carboxylase. In addition to functioning as the positive allosteric effector for site-specific DNA binding, bio-5′-AMP is the activated intermediate in biotin transfer. BirA catalyzes synthesis of the adenylate from the two substrates, biotin and ATP (Prakash & Eisenberg, 1979). Since no sequence-specific DNA binding of the apo-repressor to bioO has been observed (D. Beckett, unpublished observations), the requirement of the corepressor for DNA binding appears to be absolute.

The three-dimensional structure of apoBirA has been determined by X-ray crystallography (Wilson *et al.*, 1992). The protein is folded into three domains (Figure 1). As predicted from the sequence, as well as the phenotypes of single-site mutants in the region, a segment of the N-terminal domain folds into the helix−turn−helix motif which presumably contacts the DNA in the BirA−bioO complex. The topology of the central domain is similar to that found for src homology 2 (SH2) domains (Russell & Barton, 1993) and a subgroup of single-site mutants in this region of the protein are deficient in catalytic function (Barker & Campbell, 1981a,b). This conclusion is based on the *in vivo* growth requirement of such mutants for high biotin concentrations. In a cocrystal of the protein with the product analog, biotinyllysine, the ligand is bound to the central domain. Both biotin and bio-5′-AMP are, by inference, assumed to bind at the site where the analog is found to bind. The C-terminal

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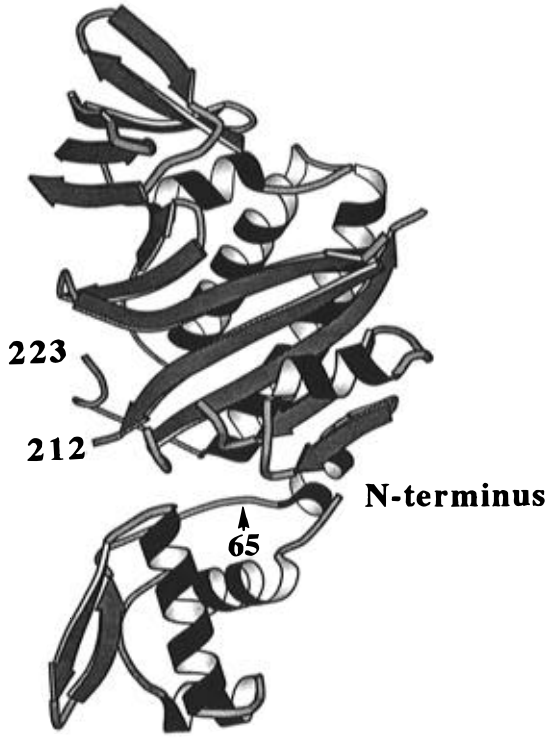

FIGURE 1: Ribbon drawing showing the topology of BirA. The termini of the unstructured loop 212−223 and the position of residue 65 are indicated by the numbers. The figure was generated using Molscript and is based on the coordinates obtained from the X-ray crystallographically-determined structure (Wilson *et al.*, 1992).

domain of the protein folds into a structure similar to that found for src homology 3 (SH3) domains (Russell & Barton, 1993). Since no mutants in this region have been characterized to date, the function of the C-terminal domain is unknown. In the three-dimensional structure of apoBirA there appears to be little interaction between the N-terminal and central domains. The biochemical data, however, indicate functional interaction between the two domains since binding of bio-5′-AMP to the central domain increases the affinity of the N-terminal domain for DNA (Eisenberg *et al.*, 1982). Binding of the adenylate to BirA is likely to result in a significant change in the conformation of BirA since diffusion of bio-5′-AMP into crystals of the apo-repressor results in cracking of the crystals (Wilson *et al.*, 1992). Results of kinetic analysis of bio-5′-AMP binding, as well as results of partial proteolysis with subtilisin in the absence and presence bio-5′-AMP, provide additional evidence for the occurrence of a significant structural transition in BirA upon binding of corepressor (Xu *et al.*, 1995). The structural change may lead to more extensive interaction between the N-terminal and central domains in the holorepressor.

In this work, we examine the effects of deletion of the N-terminal domain of BirA on the functions of the protein. As expected, the truncated protein was found to be incapable of binding to bioO. Deletion of the N-terminal domain was found to have little or no effect on the chemical step in enzyme-catalyzed synthesis of bio-5′-AMP. The rate of transfer of biotin from bio-5′-AMP to BCCP was also indistinguishable from that measured for the intact repressor. Deletion of the N-terminal domain was found to have large deleterious effects on binding of both biotin and bio-5′-AMP to the protein. The affinity of the mutant for the former ligand was decreased by 100-fold, while that for the latter ligand was decreased by 1000-fold. Detailed kinetic studies of adenylate binding to the truncated protein reveal that the decreased affinity reflects both a decrease in the bimolecular rate constant for association of the protein and ligand, as well as an increased unimolecular dissociation rate for the complex. The DNA binding domain of BirA thus appears to be functionally significant for tight binding of the corepressor to the protein. This may indicate intimate association between the two domains in the holorepressor. The interdomain interaction may have been significant for the evolution of this multifunctional protein.

MATERIALS AND METHODS

Chemicals and Biochemicals. All chemicals used in preparation of buffers were at least reagent grade. The *d*-biotin and ATP (disodium salt) were purchased from Sigma. Biotinyl-5′-AMP was synthesized using a modification of the method described in Lane *et al*, 1965 (Abbott & Beckett, 1993). Restriction enzymes were obtained from Promega and Klenow fragment was from BRL. Unlabeled deoxynucleoside triphosphates were from P. L. Biochemicals. The [α-^{32}P]deoxynucleoside triphosphates used in end-labeling of DNAs were purchased from Amersham. Wild type BirA was purified using the method described in Abbott and Beckett (1993). The C-terminal 87 residue fragment of the biotin carboxyl carrier protein (BCCP-87) was purified using the method described by E. Nenortas and D. Beckett (submitted for publication).

Bacterial Strains and Plasmids. Strain JM109 (e14$^-$*(mcrA), recA1, endA1, gyrA96, thi-1, hsdR17* (r_k^-, m_k^+)*, supE44, relA1,* Δ(lac-proAB), [F′ traΔ36, proAB, lacI^{q}ZΔM15]) has been previously described. Plasmid pBTac1 (Boehringer Mannheim Biochemicals) was used for cloning of the N-terminal truncated region of the BirA gene. The segment of the BirA gene encoding residues 65−321 was amplified off of the plasmid pJMR1 (A. J. Otsuka and J. Matsuzaki, unpublished). Plasmid pBioZ, which was the source of the DNA fragment used in DNase footprints, has been previously described (Abbott & Beckett, 1993).

*Construction of birA*65−*321.* DNA manipulations were performed as described in Maniatis *et al.* (1982). The segment of the *birA* gene encoding residues 65−321 (*birA*65−321) was amplified by PCR off of plasmid pJMR1 (A. J. Otsuka and J. Matsuzaki, unpublished) according to the instructions of the supplier of Taq polymerase (Perkin-Elmer Cetus). The sequence of the oligonucleotide used for the amplification from the 5′-end of the segment was designed so that the identity of residue 65 was changed from glutamine to methionine. The amplified gene segment was cloned as an *Eco*RI−*Hind*III fragment into pBTac1 in which its expression is controlled by the tac promoter. The recombinant plasmid was transformed into bacterial strain JM109 in which transcription of the gene was controlled by addition of IPTG to the media. The DNA sequence of the cloned *birA*65−321 was determined by the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) and found to be as expected.

Purification of the BirA65−*321*. Cultures of JM109/pBTac1*birA*65−321 in LB media (+100 μg of ampicillin/mL) were grown at 20 °C until the optical density at 600 nm was approximately 1.0. Isopropyl thiogalactoside (IPTG) was added to a final concentration of 75 μg/mL, and cell

growth was continued for 4 h. All subsequent steps in the protein purification were performed at 4 °C. Cells were harvested by centrifugation, resuspended in lysis buffer (100 mM sodium phosphate pH 7.5, 200 mM NaCl, 5% glycerol, and 0.1 mM DTT), and disrupted by sonication. The lysate was cleared by centrifugation at 13 000*g* for 30 min. A 10% (volume/volume in H_2O) solution of polyethyleneimine (Sigma) at pH 7.0 was added to the supernatant to a final concentration of 0.1%, and the mixture was stirred for 30 min. The resulting precipitate was removed by centrifugation for 30 min at 13 000*g*. A saturated solution of $(NH_4)_2SO_4$ in lysis buffer was added to the supernatant to a final concentration of 60%, and the mixture was stirred overnight at 4 °C. The $(NH_4)_2SO_4$ precipitate was pelleted by centrifugation at 13 000*g* for 30 min and resuspended in buffer 1 [100 mM sodium phosphate, pH 6.5, 5% (vol/vol) glycerol, 0.1 mM DTT]. The sample was dialyzed against the same buffer and loaded onto phosphocellulose resin (Whatman) prepared according to the manufacturer's instructions and equilibrated in buffer 1. The column was washed with buffer 1 and the protein was eluted in a linear NaCl gradient (0−0.4 M) in buffer 1. The protein eluted at a NaCl concentration of approximately 0.3 M. Fractions containing the protein were pooled and dialyzed against buffer 2 [50 mM Tris-HCl, pH 7.5 at 4 °C, 50 mM KCl, 5% (vol/vol) glycerol, and 0.1 mM DTT]. The sample was loaded onto Q-Sepharose Fastflow (Pharmacia) resin equilibrated in buffer 2. A number of protein impurities bound to this resin while BirA65−321 eluted in the wash fraction. This fraction was loaded onto CM Sepharose Fastflow (Pharmacia) resin equilibrated in the same buffer. The protein was eluted in a linear KCl gradient (0.05−0.8 M) in which it eluted at a KCl concentration of 0.3 M. The purified protein was dialyzed against buffer 3 (50 mM Tris-HCl, pH 7.5 at 4 °C, 200 mM KCl, 5% glycerol, 0.1 mM DTT) and stored in 1 mL aliquots at −70 °C. The protein was determined to be 98% pure as judged by results of SDS−PAGE analysis. Protein concentration was determined by UV absorbance using an extinction coefficient of 1.4 mL mg^{-1} cm^{-1} determined according to Gill and von Hippel (1989).

Plasmid and Biotin Operator Fragment Preparation. The fragment used for DNA binding measurements was obtained from plasmid pBioZ which was prepared from *E. coli* strain X90 according to the method of Birnboim and Doly (1979) and finally purified by CsCl density gradient centrifugation (Maniatis *et al.*, 1982). The plasmid was first restricted with the enzyme *Hind*III, labeled with ^{32}P by end filling using the large fragment of DNA polymerase I and [α ^{32}P]dNTPs and then cleaved with *Pst*I. The resulting labeled fragment was purified from an agarose gel by electroelution (Maniatis *et al.*, 1982).

DNase Footprint Titrations. DNase footprint titrations were performed as described in Brenowitz *et al.* (1986). The buffer used for measurements contained 10 mM Tris-HCl, pH 7.50 ± 0.02 at 20.0 ± 0.1 °C, 50 mM KCl, 2.5 mM $MgCl_2$, 1 mM $CaCl_2$, 2 μg of sonicated calf thymus DNA/mL, 100 μg of bovine serum albumin/mL, 50 μM biotin, and 0.5 mM ATP. The products were separated on a 10% denaturing polyacrylamide gel and then exposed to X-ray film (Kodak XAR5).

Measurement of Time Course of Bio-5′-AMP Synthesis. The time course of BirA-catalyzed synthesis of bio-5′-AMP 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 or BirA65−321, ATP, and biotin in buffer A (10 mM Tris-HCl, 200 mM KCl, and 2.5 mM $MgCl_2$, pH = 7.50 ± 0.02 at 20.0 ± 0.1 °C). The concentrations of components present in the reported measurements are indicated in Results. In order to drive the reaction equilibrium toward completion, inorganic pyrophosphatase 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 20.0 ± 0.1 °C. 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 Company). 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 vol/vol/vol). Quantitation of the bio-5′-AMP and ATP was achieved 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.

Steady-State Fluorescence Measurements. Fluorescence measurements were made using an SLM 48000 spectrofluorimeter. All measurements were performed in buffer A. 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 430 nm. The excitation slit width was set at 2 nm, and the emission slit width was 4 nm. All measurements were made in the ratio mode using rhodamine B as a quantum counter. Titrations were performed by sequential addition of small volumes of concentrated bio-5′-AMP to a solution containing protein. The mixtures were equilibrated for at least 2 min prior to measurement of the spectra. Spectra were corrected for the contribution of buffer as well as 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 of the adenosine moiety of bio-5′-AMP is zero at 295 nm.

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. Buffer was degassed by bubbling helium through it, and solutions were filtered through 0.45 mm 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 Corporation). Constant temperature was maintained by circulating water from a constant temperature bath through the stopped-flow module.

Initial Rates of Bio-5′-AMP Synthesis. The initial rate of BirA65−321-catalyzed synthesis of bio-5′-AMP from biotin and ATP was measured by stopped-flow fluorescence. One syringe contained the enzyme premixed with a large molar

excess of ATP in buffer A. The second syringe contained biotin at a range of initial concentrations. All biotin concentrations were significantly greater than the enzyme concentration. The contents of the two syringes were rapidly mixed in a 1:1 ratio, and the time-dependent decrease in the fluorescence intensity was measured.

Measurement of the Rate of Association of BirA65−321 with bio-5′-AMP. The rate of association of BirA65−321 with bio-5′-AMP was measured by stopped-flow fluorescence. The final protein concentration in the observation cell was constant for all measurements, and the bio-5′-AMP concentration was varied. The contents of the two syringes were rapidly mixed in a 1:1 ratio, and the time-dependent decrease in fluorescence intensity was monitored.

Measurement of the Rate of Dissociation of the BirA65−321-Bio-5′-AMP complex. The rate of dissociation of the BirA65−321−bio-5′-AMP complex was measured by rapidly mixing a 1:1 mixture of the protein and ligand with a large molar excess of biotin and measuring the time-dependent increase in the intrinsic protein fluorescence. The biotin concentration was sufficiently high so that the rate-determining step in the process was the release of bio-5′-AMP from the protein−ligand complex.

Measurement of the Rate of Transfer of biotin from bio-5′-AMP to the Biotin Carboxyl Carrier Protein. The apparent BirA65−321-catalyzed rate of transfer of biotin from bio-5′-AMP to BCCP87 was measured by monitoring the time-dependent increase in the intrinsic BirA65−321 fluorescence upon rapidly mixing a 1:1 complex of the enzyme−adenylate complex with the acceptor protein. The final concentration of BirA67−321 in the observation cell was 1 μM, and the BCCP87 concentration was varied.

Data Analysis. All primary stopped-flow fluorescence data were analyzed using the software provided with the instrument. Nonlinear least-squares analysis of steady-state fluorescence titration data was performed using Nonlin (Johnson & Faunt, 1992). Analysis of the dependence of the initial rate of bio-5′-AMP synthesis on biotin concentration was performed using the same program.

RESULTS

Deletion of the N-terminal domain of BirA was designed to examine the interdependence of the functions of this domain and the remainder of the protein. The choice of residue 65 as the N-terminal residue of the truncated protein was based on the X-ray crystallographically-determined high-resolution structure of the intact protein (Wilson *et al.*, 1992). This residue is located in the linker region between the N-terminal and central domains in the structure (Figure 1). Data from mutational studies (Barker & Campbell 1981a,b; Buoncristiani *et al.*, 1986), as well as the structural information clearly indicate that the N-terminal domain, a portion of which folds into the familiar helix−turn−helix motif, directly contacts the biotin operator in the protein−DNA complex. We have performed DNA binding experiments with BirA65−321 to test its ability to bind to the biotin operator sequence. Results of DNase footprints performed with the truncated and wild type protein are shown in Figure 2. The footprints were performed at a low monovalent ion concentration since binding of BirA to bioO is tighter under these conditions. As indicated in the figure, no binding of BirA65−321 to the biotin operator is observed at a protein concentration 10-fold greater than that required to fully saturate the operator with the intact repressor. We conclude that, as expected, deletion of the N-terminal domain abolishes the DNA binding function of BirA.

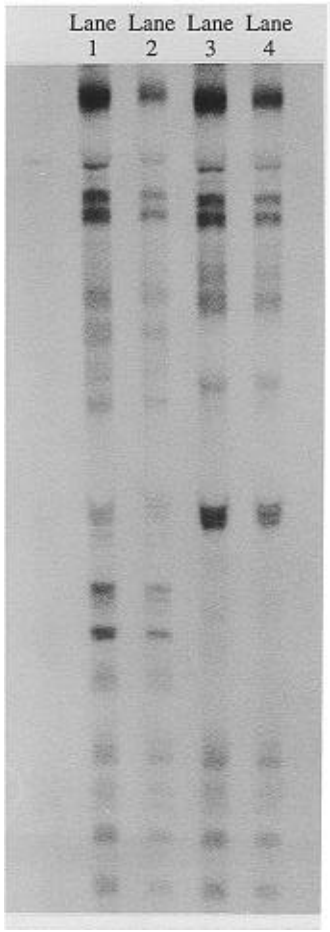

FIGURE 2: DNase footprints of the biotin operator sequence. Lane 1, no repressor; lane 2, 1×10^{-6} M BirA65−321; lane 3, 1×10^{-7} M BirA; lane 4, 1×10^{-6} M BirA. The conditions used for the binding experiments are provided in Materials and Methods.

Activity of BirA65−321. The activity of the N-terminal domain-deleted repressor in catalyzing synthesis of bio-5′-AMP from biotin and ATP was measured using a previously published method (Xu & Beckett, 1994). The method is based on quantitating the incorporation of ^{32}P from the α-phosphate of [α^{32}P]ATP into bio-5′-AMP. The substrate and product are readily separated by thin-layer chromatography on cellulose, and quantitation is accomplished by direct scanning of the cellulose plate using the Molecular Dynamics PhosphorImaging densitometer. Time courses of bio-5′-AMP synthesis catalyzed by BirA and BirA65−321 are shown in Figure 3. The time course of intact BirA-catalyzed synthesis of bio-5′-AMP is characterized by a rapid exponential burst followed by a slow linear phase (Xu & Beckett, 1994). The following chemical equation describes this process:

$$\text{BirA} + \text{biotin} + \text{ATP} \xrightarrow{k_{syn}} \text{BirA}-\text{bio-5′-AMP} \xrightarrow{k_{off}} \text{BirA} + \text{bio-5′-AM} \quad (1)$$

where the first step in the reaction, involving synthesis of bio-5′-AMP, corresponds to the rapid burst in the time course and the second step, which is rate limited by release of

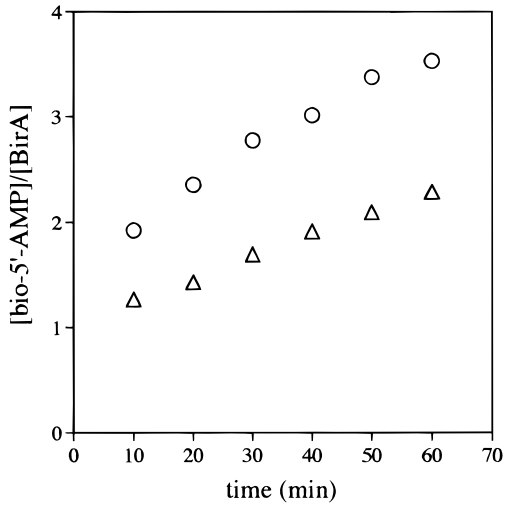

FIGURE 3: Time course of (△) BirA and (○) BirA65−321-catalyzed synthesis of bio-5′-AMP measured by quantitation of [^{32}P]bio-5′-AMP and [α ^{32}P]ATP resolved by thin-layer chromatography. [ATP] = 0.5 mM, [biotin] = 0.1 mM, and [protein] = 1 μM. The reactions were carried out in buffer A at 20 °C.

product from BirA, corresponds to the slow linear phase. The data shown in Figure 3 demonstrate that BirA65−321 is active in synthesis of bio-5′-AMP. The intercept and slope obtained from linear least-squares analysis of the [bio-5′-AMP] vs time data obtained with intact BirA provide the stoichiometry and dissociation rate constant, respectively, for the protein−bio-5′-AMP complex. This is not necessarily true for the truncated protein because use of the simple linear equation for analysis of the kinetic data is limited to cases in which the reaction follows burst kinetics (Fersht, 1985). More specifically, k_{syn} must be much greater than k_{off}, a condition that may not hold for the kinetics of adenylate synthesis catalyzed by the truncated protein. A second factor that may contribute to the observed difference in the time courses of intact and BirA65−321-catalyzed adenylate synthesis is the possibility of a significant contribution of the reverse reaction to the measured process. Pyrophosphate is a product of the reaction and we have included pyrophosphatase in the reaction mixtures to eliminate the possibility of the reverse reaction. If the dissociation rate of the BirA65−321−adenylate complex is significantly greater than that of the complex formed with intact BirA the concentration of PP_i present in the reaction catalyzed by the mutant protein would be significantly greater than that found in the mixtures containing the wild type enzyme. The amount of pyrophosphatase utilized in the reaction catalyzed by BirA65−321 may not be sufficient to prevent the reverse reaction.

The fractional activity of BirA65−321 was measured by stoichiometric titration of the protein with bio-5′-AMP. Quenching of 35% of the intrinsic protein fluorescence signal is observed upon saturation of the protein with ligand. A stoichiometric titration performed at approximately 5 μM BirA65−321 is shown in Figure 4A. Analysis of the data using a simple breakpoint model indicate a ligand to protein stoichiometry of 1.06 ± 0.02 for BirA65−321. On the basis of this result we conclude that the protein is 100% active in binding to the adenylate.

Binding of Bio-5′-AMP to BirA65−321. Binding of bio-5′-AMP to intact BirA is characterized by a high affinity, and the equilibrium dissociation constant measured in buffer A at 20 °C is 5×10^{-11} M (Xu & Beckett, 1994; Xu *et al.*,

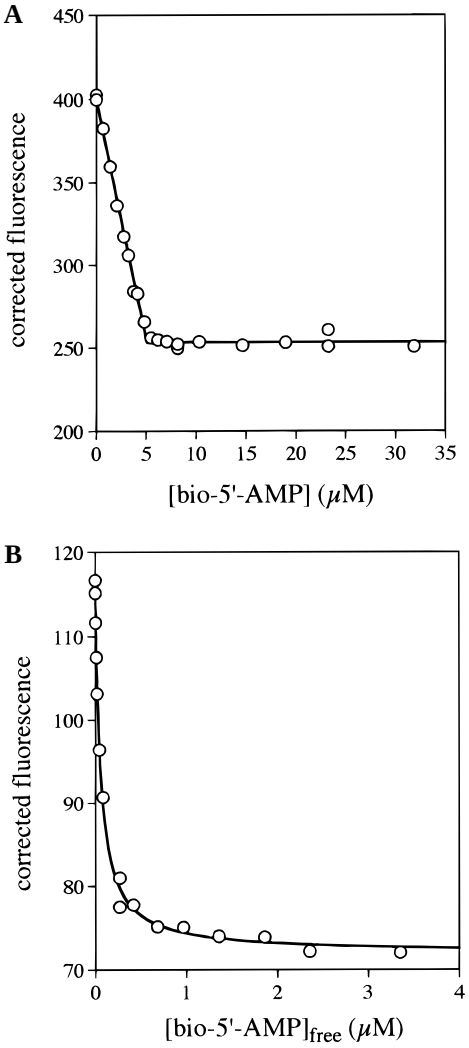

FIGURE 4: (A) Stoichiometric titration curve for BirA65−321 with bio-5′-AMP obtained from steady-state fluorescence measurements. The titration was performed in buffer A at 20 °C. The solid line represents the best-fit curve obtained from least-squares analysis of the data using a simple relation for binding of bio-5′-AMP to the protein. [BirA65−321] = 4.97 μM. (B) Equilibrium titration of birA65−321 with bio-5′-AMP obtained from steady-state fluorescence measurements. The titration was performed in buffer A at 20 °C. [BirA] = 1 μM. The solid line was simulated using the best-fit parameters obtained from nonlinear least-squares analysis of the data using a simple binding model.

1995). Equilibrium fluorescence titrations were performed to measure the affinity of bio-5′-AMP for BirA65−321, and the results are shown in Figure 4B. Analysis of the data using a simple binding model yields the equilibrium constant of 6 (± 2) × 10^{-8} M and a resulting Gibbs free energy for binding in the buffer conditions employed of −9.7 ± 0.2 kcal/mol. Deletion of the N-terminal domain of BirA results in approximately a 1000-fold decrease in the affinity of the protein for bio-5′-AMP.

The kinetics of binding of bio-5′-AMP to intact BirA are well characterized (Xu *et al.*, 1995). We have measured the kinetics of binding of BirA65−321 using stopped-flow fluorescence. Results of measurement of the bimolecular association of the protein and ligand are shown in Figure 5A. As observed with the intact repressor, the kinetics of association are biphasic and are well described by a double-exponential model. The time constant of the first phase is dependent on ligand concentration while that of the second

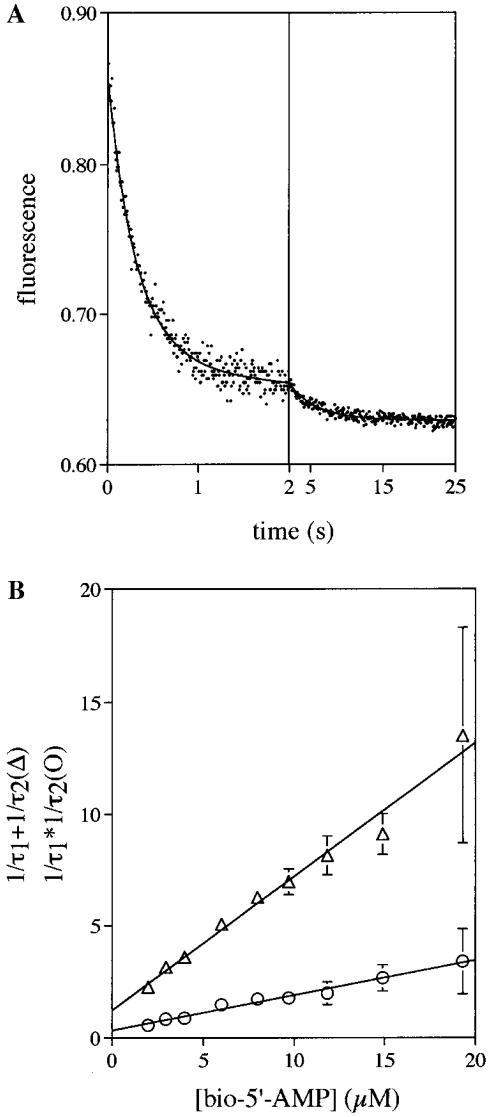

FIGURE 5: (A) Stopped-flow fluorescence trace obtained for association of BirA65-321 with bio-5′-AMP. The measurement was performed in buffer A at 20 °C. [BirA65−321] = 0.5 μM and [bio-5′-AMP] = 4 μM in the observation cell. (B) Dependence of the sum and product of the time constants for the first (τ_1) and second (τ_2) phases in association of BirA65−321 with bio-5′-AMP on ligand concentration. For points in which error bars are not shown the error is within the symbol. The solid lines were obtained using best-fit parameters obtained from weighted linear least-squares analysis of the data.

is ligand concentration independent (data not shown). Consistent with this observation, we have interpreted the kinetic data to represent initial collision of the protein and ligand, followed by a slow change in the conformation of the complex (Xu *et al.*, 1995). The reaction is modeled by a general two-step binding equation:

$$\text{BirA} + \text{bio-5′-AMP} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{BirA−bio-5′-AMP} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} {}^*\text{BirA−bio-5′-AMP} \quad (2)$$

where BirA−bio-5′-AMP represents the initial collision complex and *BirA−bio-5′-AMP is the isomerized complex. The relationships between the measured time constants, τ_1 and τ_2, and the microscopic rate constants for the binding process are given by the following expressions (Fersht, 1985):

$$\frac{1}{\tau_1} + \frac{1}{\tau_2} = k_1[\text{bio-5′-AMP}] + k_{-1} + k_2 + k_{-2} \quad (3)$$

$$\frac{1}{\tau_1} \times \frac{1}{\tau_2} = k_1(k_2 + k_{-2})[\text{bio-5′-AMP}] + k_{-1}k_{-2} \quad (4)$$

Results of analysis of the association data using these equations are shown in Figure 5B. The microscopic rate constants obtained from the data are shown in Table 1. The value of k_1 for binding of bio-5′-AMP to BirA65−321 is 10-fold lower than that measured for the intact protein (Xu *et al.*, 1995).

The rate of dissociation of the BirA65−321−bio-5′-AMP complex was also measured by stopped-flow fluorescence. A 1:1 complex of the protein and ligand was rapidly mixed with a large molar excess of biotin and the time-dependent increase in the intrinsic protein fluorescence due to release of bio-5′-AMP was monitored (Figure 6). The data are well described by a single-exponential model, and the best-fit unimolecular dissociation rate constant obtained from analysis of the data is 0.044 ± 0.002 s^{-1}. This value is 100-fold greater than that measured for the BirA−bio-5′-AMP complex (Xu & Beckett, 1994).

Table 1: Microscopic Rate Constants for Binding of Bio-5′-AMP to BirA and BirA65-321

	k_1 ($M^{-1}s^{-1}$)	$k_2 + k_{-2}$ (s^{-1})	k_{-1} (s^{-1})
BirA[a]	5.9 (±0.8) × 10^6	0.32 ± 0.09	1 ± 3[b]
BirA65−321[c]	6 (±1) × 10^5	0.27 ± 0.09	0.9 ± 0.5

[a] Values from Xu *et al.* (1995). [b] A more precise value of k_{-1} for intact BirA obtained from direct measurement of partitioning of the intermediate complex is approximately 0.3 s^{-1} (Xu *et al.*, 1995). [c] Values were obtained from weighted linear least- squares analysis of the kinetic data using eqs 3 and 4.

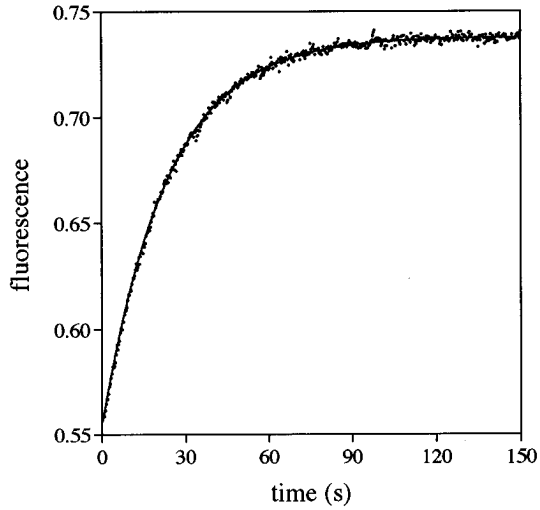

FIGURE 6: Stopped-flow fluorescence trace obtained for measurement of the dissociation of the BirA65−321−bio-5′-AMP complex. A 1:1 complex of BirA65−321−bio-5′-AMP was rapidly mixed with a large molar excess of biotin, and the resultant time-dependent increase in intrinsic protein fluorescence was measured. $[\text{BirA}]_{\text{final}}$ = $[\text{bio-5′-AMP}]_{\text{final}}$ = 0.5μM; $[\text{biotin}]_{\text{final}}$ = 420 μM. The solid line was simulated using the best-fit parameters obtained from nonlinear least-squares analysis of the data using a single-exponential model. The measurement was performed in buffer A at 20 °C.

The measured kinetic data for binding of BirA65−321 to bio-5′-AMP can be used to estimate the equilibrium dissociation constant for the binding process. The bimolecular association rate constant, k_{on}, and unimolecular dissociation rate constant, k_{off}, are related to the equilibrium dissociation constant or K_D using the familiar relationship, $K_D = k_{off}/k_{on}$. For a two-step binding reaction the K_D is related to the microscopic rate parameters for binding by the following expression:

$$K_D = \frac{k_{-1}k_{-2}}{k_1 k_2} \quad (5)$$

The equilibrium dissociation constant was calculated from the microscopic rate parameters governing binding of bio-5′-AMP to BirA65−321. On the basis of the measured value of the unimolecular dissociation constant for the complex and the value of k_{-1} determined from analysis of the association rate data, the value of k_{-2} used in this calculation is 0.044 s^{-1}. The value of the constant k_2 was calculated using this value of k_{-2} and the value for the sum of the constants ($k_2 + k_{-2}$) in Table 1. The equilibrium dissociation constant calculated from the kinetic data is 3(±2) × 10^{-7} M and the resulting Gibbs free energy is −8.8 ± 0.4 kcal/mole. These values are in reasonable agreement with those obtained from direct equilibrium titrations of the protein.

Biotin Binding. Saturation of BirA with biotin results in quenching of 15% of the intrinsic protein fluorescence. By contrast, binding of biotin to BirA65−321 results in no fluorescence change and the binding could, therefore, not be measured by direct equilibrium titration. An alternative method for determining the apparent affinity of biotin for the protein is to measure the dependence of the initial rate of enzyme-catalyzed synthesis of bio-5′-AMP on biotin concentration. The initial rate of bio-5′-AMP synthesis was monitored by stopped-flow fluorescence. As indicated above, binding of bio-5′-AMP to BirA65−321 results in quenching of 35% of the intrinsic protein fluorescence signal. A solution of the protein combined with a large molar excess of ATP was rapidly mixed with biotin, and the time-dependent decrease in intrinsic protein fluorescence was monitored (Figure 7). The time course is well described by a single-exponential model. The biotin concentration dependence of the reaction was measured, and the results were analyzed using the following equation to obtain the K_M for biotin:

$$k_{app} = \frac{k[\text{biotin}]}{K_M + [\text{biotin}]} \quad (6)$$

where k is the initial rate of synthesis at saturating biotin concentration and K_M is the Michaelis constant for biotin. Results of the analysis (Figure 7) yield a K_M of 6.4 (± 0.5) × 10^{-6} M. Assuming that the K_M reflects the true equilibrium dissociation constant for binding of biotin to the protein, the affinity of BirA65−321 for biotin is 100-fold weaker than that of the intact protein for the ligand (Xu *et al.*, 1995). At 1 mM ATP the initial rate of bio-5′-AMP synthesis catalyzed by the N-terminal truncated BirA at saturating biotin concentration is 0.118 ± 0.003 s^{-1}. This value is approximately one-half of the corresponding value, 0.286 s^{-1}, measured for intact BirA.

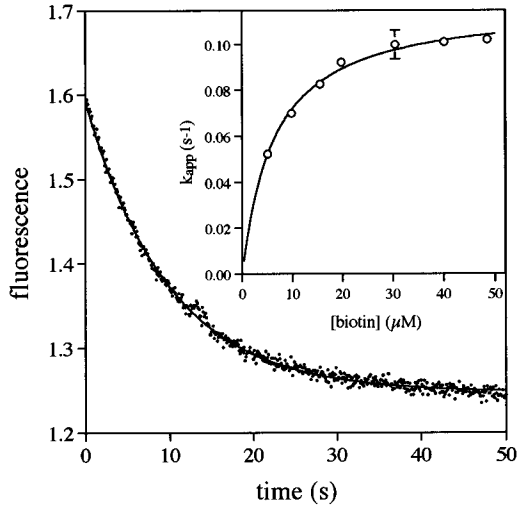

FIGURE 7: Stopped-flow fluorescence trace obtained for measurement of the initial rate of bio-5′-AMP synthesis from biotin and ATP. $[BirA]_{final}$ = 1.03 μM; $[biotin]_{final}$ = 20 μM; $[ATP]_{final}$ = 1 mM. The reaction was carried out in buffer A at 20 °C. Inset: Dependence of the initial rate of BirA65−321-catalyzed bio-5′-AMP synthesis, k_{app}, on biotin concentration.

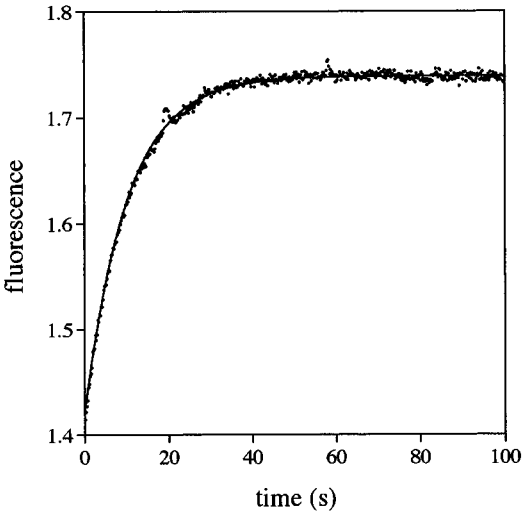

FIGURE 8: Stopped-flow fluorescence trace obtained for measurement of transfer of biotin from bio-5′-AMP to BCCP-87. A 1:1 complex of BirA65−321 was rapidly mixed with a molar excess of BCCP-87 and the time-dependent increase in the intrinsic BirA65−321 fluorescence was monitored. The solid line was simulated using the best-fit parameters obtained from nonlinear least-squares analysis of the data using a single-exponential model. The measurement was performed in buffer A at 20 °C.

Transfer of Biotin from BirA65−321−Bio-5′-AMP to apoBCCP. The rate of BirA65−321-catalyzed transfer of biotin from bio-5′-AMP to a lysine residue of the C-terminal domain of biotin carboxyl carrier protein (BCCP87) subunit of the acetyl-CoA carboxylase was measured by stopped-flow fluorescence. We have previously shown that this domain behaves identically to the intact subunit in the biotin transfer reaction (E. Nenortas and D. Beckett, submitted for publication). A 1:1 complex of BirA65−321 and bio-5′-AMP was rapidly mixed with a molar excess of BCCP87, and the resulting time-dependent increase in the intrinsic protein fluorescence was monitored (Figure 8). The data are well described by a single-exponential model, and the rates of transfer determined over a range of BCCP87 concentration from 5 to 20 μM were identical for the intact and truncated forms of BirA.

DISCUSSION

The three-dimensional structure of the repressor of biotin biosynthesis indicates that it is folded into three domains (Figure 1). A segment of the N-terminal domain folds into the helix−turn−helix motif, and results of *in vivo* studies of single-site mutants in this region provide strong evidence for a direct role of this domain in contacting the biotin operator DNA in the protein−DNA complex. The central domain folds into a structure topologically similar to that of the src homology 2(SH2) domain (Russell & Barton, 1993), and in the available cocrystal structure, biotinyllysine is found to bind to the central domain. Some single-site mutants in this domain are compromised in their ability to catalyze the covalent linkage of biotin to BCCP. The C-terminal domain of BirA folds into a structure that is reminiscent of the src homology 3 (SH3) domains (Russell & Barton, 1993). No mutations in this region have thus far been isolated. A great deal of evidence exists to indicate interdependence of the functions of the N-terminal and central domains of BirA. Some single-site mutants in the central domain have deleterious effects on the ability of BirA to repress transcription *in vivo*. A subset of these mutations is near the binding site for biotin derivatives and, presumably, for bio-5′-AMP. Since bio-5′-AMP is the positive allosteric effector for site-specific DNA binding, the mechanistic basis for the loss of repressor function in these mutants may lie in either a lower affinity for the adenylate or a decoupling of effector binding from DNA binding. The influence of the N-terminal domain on the functions of the remainder of the repressor is unknown, and the N-terminal deletion mutant was constructed, overproduced, and purified in order to address this question.

The BirA65−321 mutant was found to be inactive in binding to the biotin operator sequence. This was expected since, as discussed above, the N-terminal domain contains the helix−turn−helix motif. The mutant and intact protein were found to be equally active in catalyzing the synthesis of bio-5′-AMP from the substrates biotin and ATP. This conclusion is based on the measured maximal rates of bio-5′-AMP synthesis. The activities of both proteins are also the same in catalyzing the transfer of biotin from bio-5′-AMP to the C-terminal domain of the biotin carboxyl carrier protein. Thus, for some of its functions, the C-terminal 257 residue segment of BirA appears to be independent of the N-terminal domain of the protein.

The affinities of BirA65−321 for both biotin and bio-5′-AMP are 2 and 3 orders of magnitude, respectively, lower than those of the intact protein for the two ligands. Direct measurements of binding of biotin to the truncated protein were not performed because no change in the intrinsic protein fluorescence occurred upon saturation of the protein with the ligand. The apparent affinity of BirA65−321 for biotin was, instead, estimated from the dependence of the initial rate of bio-5′-AMP synthesis on biotin concentration. The measured K_M for biotin is assumed to reflect the true equilibrium dissociation constant for binding of the ligand. This assumption is justified by the low value of the maximal rate of synthesis of the adenylate from biotin and ATP which indicates that the chemical step is rate limiting in the synthesis of bio-5′-AMP. The K_D for binding of the intact repressor to biotin obtained from direct equilibrium fluorescence titrations as well as independent kinetic measurements (Xu *et al.*, 1995) is approximately 4×10^{-8} M. No dependence of the initial rate of bio-5′-AMP synthesis on biotin concentration is observed for the intact repressor in the micromolar range of substrate concentration. On the basis of this observation we have concluded that the K_M of the intact repressor for biotin is in the submicromolar range and, like the truncated protein, may be identical to the K_D for binding of the substrate.

The K_D for bio-5′-AMP binding to BirA65−321 is 3 orders of magnitude greater than that measured for the intact repressor-bio-5′-AMP interaction. Comparison of the detailed kinetics of binding of the adenylate to the two proteins indicates that this decreased affinity results from both a decrease in the bimolecular rate of association of the protein and ligand and an increase in the dissociation rate of the complex. The kinetics of association of bio-5′-AMP with BirA65−321 are biphasic. As with the intact repressor (Xu *et al.*, 1995) we have interpreted this to indicate initial formation of a collision complex followed by a slow conformational change in the complex. The bimolecular rate constant, k_1, governing formation of the collision complex is 10-fold smaller for the truncated protein than for intact BirA. The measured value, 6×10^5 M^{-1} s^{-1}, is considerably smaller than that predicted for a diffusion-controlled process. This is not unusual (Simpopolous & Jencks, 1994) and may reflect, for example, specific orientation requirements for productive binding. The 10-fold decrease in the value of k_1 that results from deletion of the N-terminal domain may be due to an increased flexibility in the region of the protein that defines the bio-5′-AMP binding site. Exposure of BirA to subtilisin results in initial cleavage of the protein between residues 217 and 218 in the unstructured loop defined by residues 212−223. As discussed above, this loop is in the vicinity of the putative binding site for bio-5′-AMP. The rate of proteolytic digestion of BirA65−321 is dramatically increased relative to that observed for the intact protein (data not shown), indicating that the loop is more accessible to proteolysis in the truncated protein and may be more flexible. The decreased rate of formation of the collision complex observed for binding of bio-5′-AMP to the truncated protein may reflect this increased flexibility. The rate of dissociation of BirA65−321−bio-5′-AMP is 100-fold faster than that measured for BirA−bio-5′-AMP. This relatively fast dissociation rate provides an explanation for why the time courses of synthesis of bio-5′-AMP from biotin and ATP differ significantly for the truncated and wild type enzymes. The difference in the magnitudes of k_{syn} and k_{off} in eq 1 for the truncated enzyme is only 2.5-fold for the BirA mutant and, consequently, the reaction does not follow burst kinetics. Moreover, as indicated in the results section, the fast rate of dissociation of the BirA65−321 complex leads to high pyrophosphate concentrations in the reaction mixtures and the possibility of significant contribution of the reverse reaction to the measured time course. Analysis of the detailed binding kinetics indicate that the rate constants for release of bio-5′-AMP from the intermediate complex, k_{-1}, are similar for truncated and intact BirA. On the basis of the measured values of k_{off} (see Results) and the values of $(k_2 + k_{-2})$ for the two proteins, the isomerization step is the slower in the overall dissociation of the complexes of each protein with bio-5′-AMP. The value of k_{-2} is, however, approximately 100-fold greater for BirA65−321 than for BirA. This result indicates that the N-terminal domain is

crucial for the kinetic stability of the BirA−bio-5′-AMP complex.

The most striking consequence of deletion of the N-terminal domain of BirA for the activity of the remainder of the protein is the dramatic decrease in the affinity of the protein for the two ligands, biotin and bio-5′-AMP. The three-dimensional structure of BirA, combined with results of partial proteolytic digestion of apo and ligand bound forms of BirA, provides a possible structural explanation for this dramatic loss of binding free energy. Four unstructured regions of the polypeptide chain are found in the central domain of the protein. Two of these loops, one including residues 116−124 and the other 212−223, are in the vicinity of the binding site for the biotin derivative, biotinyllysine (Wilson *et al.*, 1992). Phenotypes of single-site mutants in this region indicate that it is also the site for biotin and bio-5′-AMP binding (Barker & Campbell 1981a,b; Howard *et al.*, 1985; Buoncristiani *et al.*, 1986). Exposure of BirA to subtilisin results in initial cleavage of the protein at the peptide bond between residues 217 and 218 (Xu *et al.*, 1995) in the second loop and a decreased rate of proteolytic cleavage at this site is observed when the protein is saturated with biotin or bio-5′-AMP. In the structure of the protein (Figure 1) the N-terminal domain is positioned so that a shift in the orientation of this domain relative to the central domain could result in tight packing of the N-terminal domain against loop 212−223. A close association of the two domains in the liganded protein is consistent with the measured decrease in the rate of proteolysis of the loop region. Biotin and bio-5′-AMP may bind with reduced affinity to the truncated repressor because removal of the N-terminal domain results in loss of the stabilizing energy provided by the interdomain interaction.

The loss of affinity of BirA for biotin and bio-5′-AMP resulting from deletion of the N-terminal domain is particularly intriguing in light of the multiple functions of the protein. BirA belongs to a class of enzymes, the biotin holoenzyme synthetases or biotin ligases, that catalyze covalent ligation of biotin to biotin-dependent carboxylases. Since these carboxylases are found across evolution, the biotin ligases are also found in organisms ranging from bacteria to humans. Among the biotin ligases, only a subgroup are also transcriptional repressors. Biotin is a substrate for BirA-catalyzed synthesis of bio-5′-AMP, and the adenylate is the activated intermediate in the biotin transfer reaction as well as the positive allosteric effector for sequence-specific DNA binding. We have previously proposed that the high affinity of BirA for bio-5′-AMP allows for stringent coupling of the ligase and transcriptional repressor functions of the protein. This high affinity ensures that the most probable intracellular form of BirA is the adenylate-bound form. Consequently, the protein is always in the active form required for either biotin ligation or transcriptional repression. Once the cellular requirement for biotin is fulfilled the protein binds to bioO to repress transcription of the biotin biosynthetic genes. The reduced affinity of BirA65−321 for bio-5′-AMP indicates that the DNA binding domain of BirA is also required for tight binding of the adenylate. The protein appears to have evolved so that a distinct module that requires adenylate binding for its function also ensures tight binding of the adenylate. It would be interesting to compare BirA to the class of biotin holoenzyme synthetase that do not bind to DNA with respect to their affinities for the adenylate.

Modulation of the affinity of a transcriptional regulatory protein for its target sequence provides a general mechanism for response of a biological system to its environment. Structural and thermodynamic features of binding of effectors to several transcriptional regulatory proteins have been studied. Among the well-characterized systems are the *E. coli* lac repressor, the cAMP receptor protein (CRP), and the tryptophan repressor. The tryptophan repressor is an example of a protein in which the effector or corepressor is intimately involved in the sequence-specific DNA binding interaction and is found at the protein−DNA interface. In both the lac repressor and CRP, the effector binding sites are physically distinct from the DNA binding domains (Platt *et al.*, 1973; Eilen *et al.*, 1978; Aiba & Krakow, 1981). Truncated forms of both of these proteins, in which the DNA binding domain has been deleted, have been studied with respect to effector binding. The affinity of the lac repressor core domain for IPTG is indistinguishable from that of the intact repressor for effector (Platt *et al.*, 1973). The N-terminal domain of CRP obtained from chymotrypsin-catalyzed cleavage of the intact protein is altered in its ability to bind cyclic AMP (Heyduk *et al.*, 1992). Only one site in the dimer binds effector and its affinity is increased approximately 10-fold relative to that of the first site in intact CRP for cAMP. BirA is also an example of a transcriptional regulatory protein in which the effector and DNA binding domains are found on distinct structural domains. In the three-dimensional structure of apoBirA, the interaction between the two domains is tenuous. The dramatic loss of affinity of BirA for the effector resulting from deletion of the N-terminal domain indicates, however, that the DNA binding domain actively participates in tight binding of the effector. We infer from this result that closer association between the two domains exists in the holorepressor. This interdomain interface appears to be critical for the linkage of corepressor and site-specific DNA binding in BirA.

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REFERENCES

Abbott, J., & Beckett, D. (1993) *Biochemistry 32*, 9649−9656.
Aiba, H., & Krakow, J. (1981) *Biochemistry 20*, 4774−4780.
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.
Birnboim, H., & Doly, J. (1979) *Nucleic Acids Res. 7*, 1513−1523.
Buoncristiani, M. R., Howard, P. K., & Otsuka, A. J. (1986) *Gene 44*, 255−261.
Brenowitz, M., Senear, D. F., Shea, M. A., & Ackers, G. K. (1986) *Methods Enzymol. 130*, 132−181.
Cronan, J. E., Jr. (1989) *Cell 58*, 527−529.
Eilen, E., Pampeno, C., & Krakow, J. (1978) *Biochemistry 17*, 2469−2473.
Eisenberg, M. A., Prakash, O., & Hsiung, S.-C. (1982) *J. Biol. Chem. 257*, 15167−15173.
Fersht, A. (1985) *Enzyme Structure and Mechanism*, pp 135−136, W. H. Freeman and Company, New York.
Gill, S. C., & von Hippel, P. H. (1989) *Anal. Biochem. 182*, 319−326.
Heyduk, E., Heyduk, T., & Lee, J. C. (1992) *Biochemistry 31*, 3682−3688.

Howard, P. K., Shaw, J., & Otsuka, A. J. (1985) *Gene 35*, 321−331.
Johnson, M. J., & Faunt, L. M. (1992) *Meth. Enzymol. 210*, 1−37.
Lane, M. D., Rominger, K. L., Young, D. L., & Lynen, F. (1964) *J. Biol. Chem. 239*, 2865−2871.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
McKay, D., Weber, I., & Steitz, T. (1982) *J. Biol. Chem. 257*, 9518−9524.
Otsuka, A., & Abelson, J. (1978) *Nature 276*, 689−693.
Platt, T., Files, J. G., & Weber, K. (1973) *J. Biol. Chem. 248*, 110−121.
Prakash, O & Eisenberg, M. A. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5592−5595.
Russell, R. B., & Barton, G. J. (1993) *Nature 364*, 765.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5487.
Simopoulis T. T., & Jencks, W. P. (1994) *Biochemistry 33*, 10375−10380.
Wilson, K. Shewchuk, L. M., Otsuka, A. J., & Matthews, B. W. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 9257−9261.
Xu, Y., & Beckett, D. (1994) *Biochemistry 33*, 7354−7360.
Xu, Y., Nenortas, E., & Beckett, D. (1995) *Biochemistry 34*, 16624−16631.

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