Evidence for Distinct Ligand-Bound Conformational States of the Multifunctional *Escherichia coli* Repressor of Biotin Biosynthesis†

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ABSTRACT: The *Escherichia coli* repressor of biotin biosynthesis (BirA) is a unique transcriptional repressor which catalyzes synthesis of its own corepressor and catalyzes attachment of a cofactor to an essential metabolic enzyme. BirA both catalyzes synthesis of biotinyl-5′-AMP from the substrates ATP and biotin and transfer of the biotin moiety from the adenylate to a lysine residue of a subunit of the acetyl-CoA carboxylase. BirA−bio-5′-AMP, moreover, binds sequence specifically to the biotin operator to repress transcription of the biotin biosynthetic genes. Using a combination of kinetic measurements of binding of the two ligands, biotin and bio-5′-AMP, to BirA as well as proteolytic digestion experiments, we have found evidence for at least three discrete conformational states of BirA. Results of stopped-flow fluorescence measurements of association of both ligands with BirA indicate that the process involves initial formation of a collision complex followed by a slow conformational change. The kinetics of the conformational change are distinct for the two ligands and are the basis for the difference in the thermodynamic stabilities of the two protein−ligand complexes. Different rates of proteolytic digestion of apoBirA and complexes of BirA with the two ligands were also observed. Results of the combined approaches indicate that apoBirA, and the BirA−bio-5′-AMP and BirA−biotin complexes are conformationally distinct.

BirA is a 35.3 kDa *Escherichia coli* protein that possesses both enzymatic and sequence-specific DNA binding activities (Cronan, 1989; Barker & Campbell, 1981a,b) (Figure 1). Its enzymatic functions include catalysis of synthesis of biotinyl-5′-adenylate (bio-5′-AMP) and catalysis of transfer of biotin from the adenylate to a lysine residue of the biotin carboxyl carrier protein (BCCP) of the acetyl CoA-carboxylase. BirA also binds site specifically to the 40 base pair biotin operator sequence (bioO) to repress transcription of the biotin biosynthetic genes. In addition to serving as the intermediate in the biotin transfer reaction, bio-5′-AMP is the positive allosteric effector for site-specific DNA binding (Eisenberg et al., 1982). BirA, thus, both funnels biotin into metabolism via its biotin ligase function and regulates the intracellular biotin concentration via its sequence-specific DNA binding function.

BirA represents an example of remarkably efficient use of an amino acid sequence of limited size. Its 321 residues code for binding of several small ligands including the substrates biotin and ATP, the enzymatic intermediate, bio-5′-AMP, and the products inorganic phosphate and AMP. Residues encoding binding of several large molecules are also present in the sequence. These include those required for sequence-specific DNA binding, for homologous dimerization, and for the heterologous protein−protein interaction formed with BCCP. Residues required for enzymatic catalysis are also present in the BirA sequence. Regulation of the multiple functional states of BirA may be achieved via conformational transitions in the protein. Three-dimensional structures of two forms of the protein, apoBirA and a complex of BirA with biotinyllysine, have been determined by X-ray crystallographic techniques (Wilson et al., 1992). These structural data indicate that only a minor conformational change, involving ordering of three residues in a segment of the polypeptide chain that is disordered in the aporepressor structure, occurs in BirA upon binding of biotinyl-lysine. Exposure of either biotin or bio-5′-AMP to BirA crystals results in cracking of the crystals (Wilson et al., 1992). The conformations of BirA in its other complexes are, therefore, likely to differ significantly from those observed in the two known structures. BirA is an allosteric DNA-binding protein with bio-5′-AMP serving as the positive effector. The aporepressor exhibits no sequence-specific DNA binding activity (D.B., unpublished results). Binding of the allosteric effector may shift the protein to an alternate conformation that is active in DNA binding. BirA

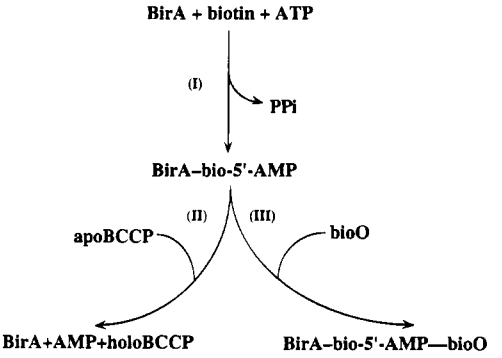

FIGURE 1: Schematic representation of the functions of the repressor of biotin biosynthesis (BirA). The protein (I) catalyzes synthesis of biotinyl-5′-adenylate (bio-5′-AMP) from the substrates biotin and ATP, (II) catalyzes transfer of biotin from the adenylate to a lysine residue of the biotin carboxyl carrier protein (BCCP) subunit of acetyl-CoA carboxylase, and (III) binds sequence specifically to the biotin operator sequence.

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also binds biotin and ATP, the substrates for bio-5′-AMP synthesis. Structural transitions in the protein may accompany substrate binding or other steps in this enzymatic reaction. BirA forms a complex with the BCCP subunit in the second step of the biotin transfer reaction, and the detailed conformation of BirA in this protein−protein complex may differ from those of the protein in its other functional states. The availability of structural information relevant to the multiple functional states of BirA will prove invaluable for understanding regulation of function in this unique protein.

In this work we present results of kinetic measurements of binding of biotin and bio-5′-AMP to BirA. Stopped-flow fluorescence measurements of association of the two ligands with BirA indicate that for both ligands the kinetics are complex and well described by a double-exponential model. The first phase is rapid and dependent on ligand concentration, while the second is slow and independent of ligand concentration. These results are consistent with a model in which an initial rapid collisional event occurs followed by a slow conformational change in the complex. The rate constants governing the conformational change are quantitatively very different for the two ligands and are the kinetic basis for a 4 kcal/mol difference in the binding affinity of BirA for biotin and bio-5′-AMP. In addition, partial proteolysis with subtilisin has been used to probe the conformations of apoBirA and its complexes with biotin and bio-5′-AMP. These measurements indicate that the susceptibility of the protein to proteolytic digestion depends on ligation state. The combination of results of kinetic measurements of binding and proteolytic digestion provide evidence for three conformational states of the multifunctional repressor of biotin biosynthesis.

MATERIALS AND METHODS

Chemicals and Biochemicals

All chemicals used in the preparation of buffers were reagent grade. D-Biotin was purchased from Sigma. Bio-5′-AMP was synthesized and BirA was purified as previously described (Abbott & Beckett, 1993).

Stopped-Flow Fluorescence Measurements

Measurement of the Rates of Association of BirA with Ligands. The apparent rates of association of bio-5′-AMP and biotin with BirA were measured using a KinTek stopped-flow instrument (Model 2001). Experiments were performed in buffer A (10 mM Tris-HCl, 200 mM KCl, 2.5 mM $MgCl_2$, pH 7.50 ± 0.01 at 20.0 ± 0.1 °C). All buffers and solutions were degassed using helium and filtered through 0.45-μm Acrodisc PTFE filters (Gelman Sciences) before use. The final concentration of BirA in the observation cell was constant for all measurements and the concentration of ligand (either biotin or bio-5′-AMP) was varied. The BirA concentration used in all kinetic experiments was sufficiently low so that the protein was 100% monomeric in the presence and absence of ligand (Abbott & Beckett, 1993; Eisenstein and D.B., unpublished observations). The excitation wavelength was set at 295 nm, and the fluorescence emission was monitored above 340 nm using a cutoff filter (Corion Corp.). Constant temperature was maintained by circulating water from a constant temperature bath through the stopped-flow module.

Measurement of the Rate of Dissociation of the BirA−Biotin Complex. The rate of dissociation of the BirA−biotin complex was measured by stopped-flow fluorescence. A solution of the BirA−biotin complex in buffer A was rapidly combined with a solution containing a high concentration of bio-5′-AMP, and the resulting time-dependent decrease in the intrinsic BirA fluorescence was measured. The initial concentrations of BirA and biotin were equimolar and sufficiently high so that the protein was saturated with ligand. The bio-5′-AMP concentration was adjusted to a sufficiently high level so that the rate-determining step in the process was the release of biotin from the BirA−biotin complex. Excitation and emission settings were identical to those used for measurement of the bimolecular rate of association of the ligands with BirA.

Measurement of Partitioning of the BirA−Ligand Intermediate Complexes. Partitioning of the intermediates was measured by first allowing the protein and ligand (either biotin or bio-5′-AMP) to react for a sufficient time to maximally populate the intermediate (approximately 10 half-lives of the first kinetic process) followed by rapid mixing of the competing ligand (biotin or bio-5′-AMP). Data collection was initiated immediately following the second mixing step. All reactions were performed in buffer A, and instrument settings were identical to those described for the measurement of the rates of association of BirA with ligands.

Proteolysis

All BirA samples subjected to subtilisin-catalyzed proteolysis were first incubated for 30 min at 20 °C in buffer A either in the absence or presence of saturating concentrations of biotin and bio-5′-AMP. Preparative cleavage of BirA to determine the site of subtilisin catalyzed cleavage was performed in the absence of small ligand. A solution containing 14 μM BirA and 2 μg/mL subtilisin (Boehringer Mannheim) in buffer A was incubated for 2 h at 20.0 ± 0.1 °C. The reaction was quenched by addition of 100 mM phenylmethanesulfonyl fluoride (PMSF) (Sigma) in 100% ethanol to a final concentration of 12.5 mM. Cleavage products were separated by SDS−polyacrylamide gel electrophoresis (SDS−PAGE) and then transferred by electroblotting onto PVDF-immobilon-P transfer membrane (Millipore) according to the method described in LeGendre and Matsudaira (1989). Bands were visualized by Coomassie Brilliant Blue R250 staining of the membrane, and membrane fragments containing the proteolytic products were excised and subjected directly to N-terminal sequencing by automated Edman degradation at the Protein Sequencing Laboratory of the Medical Biotechnology Center at the University of Maryland at Baltimore. Four cycles were sufficient to identify the cleavage point. The total quantity of each proteolytic fragment subjected to sequencing was approximately 2−3 μg.

Analytical cleavage reactions to determine the apparent rates of subtilisin-catalyzed cleavage were measured by addition of subtilisin, freshly prepared in buffer A, to a BirA solution so that the final weight ratio of BirA to subtilisin was 33:1 and incubation was continued at 20.0 ± 0.1 °C. For experiments in which BirA was complexed to a small ligand, a sufficient amount of the ligand was present to fully saturate the protein. All proteolytic digestion measurements were performed at a BirA concentration at which the protein was 100% monomeric in the absence and presence of ligand. Ten-microliter aliquots were removed at the indicated times and quenched into 1 μL of 100 mM PMSF freshly prepared

in 100% ethanol. Six microliters of Laemmli sample buffer were added to each sample, and the samples were electrophoresed on a 15% SDS−polyacrylamide gel. Protein bands were stained with Coomassie Brilliant Blue, and the amount of intact BirA in each lane was quantitated by scanning of the wet gel using a Molecular Dynamics Laser Scanning Personal Densitometer. Independent control experiments were performed to ensure that the response of the instrument is linear over the range of quantities of intact BirA observed in the experiments.

Control experiments were performed to ascertain the effects of high concentrations of the small ligands, biotin and bio-5′-AMP, on the catalytic activity of subtilisin. This was accomplished using the synthetic chromogenic substrate *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Boehringer Mannheim) (DelMar et al., 1979). Rates of cleavage of this substrate can be monitored by measurement of the change in absorbance at 410 nm. For all small ligands used, no effect of the ligand on the rate of subtilisin-catalyzed cleavage of the chromogenic substrate was observed.

Data Analysis

Primary data from pre-steady-state kinetic measurements of biotin and bio-5′-AMP binding to BirA were analyzed by nonlinear least-squares methods using the software supplied with the stopped-flow instrument. Models used in the analysis are discussed in Results. Weighted linear least-squares analysis of the reduced data was performed using GraFit (Erithacus Software, Ltd., Staines, U.K.).

Rates of subtilisin-catalyzed cleavage of BirA were obtained by modeling the time-dependent decrease in the quantity of BirA as a pseudo-first-order process. The apparent rates of proteolysis were estimated from linear least squares analysis of ln[(total optical density of intact BirA at time = *t*)/(optical density of BirA at time = 0)] vs time data. The absolute values of the slopes were used to represent the apparent rate constants of proteolysis.

RESULTS

Kinetics of Association of BirA with Biotin and Bio-5′-AMP. Binding of biotin and bio-5′-AMP to BirA results in quenching of 15% and 40%, respectively, of the intrinsic protein fluorescence signal (Xu & Beckett, 1994). The bimolecular rate of association of each ligand with BirA can, thus, be monitored by measuring the time-dependent change in fluorescence that occurs upon mixing the protein and ligand. A stopped-flow fluorescence trace obtained from measurement of the association of bio-5′-AMP with BirA is shown in Figure 2. Since the concentrations of both protein and ligand used in the experiment are several orders of magnitude greater than the estimated equilibrium dissociation constant for the binding process (Xu & Beckett, 1994), we are confident that at equilibrium the protein is saturated with ligand. The ligand concentrations which ranged from 5−25-fold greater than the protein concentration are, moreover, sufficiently high so that conditions can be considered pseudo first order in ligand concentration. The time course of binding of bio-5-AMP to BirA is characterized by two phases. While the first rapid phase is complete in the millisecond time scale, the second slow phase requires 5−10 s for completion. The excellent agreement between the best fit curves obtained by simulation, and the experimental data indicate that the process is well described by the following

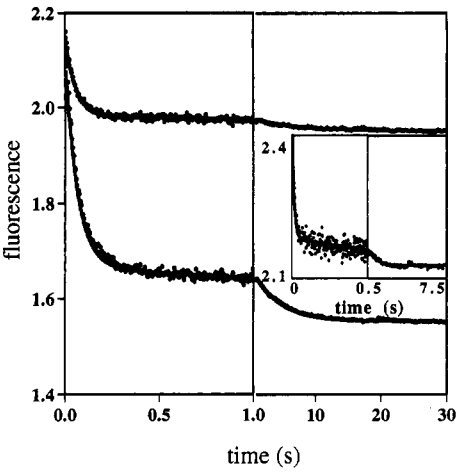

FIGURE 2: Stopped-flow fluorescence traces obtained for measurement of the bimolecular association of bio-5′-AMP (lower trace) and biotin (upper trace) with BirA. The inset shows a trace obtained for biotin binding at a higher BirA concentration. Lower and upper traces, [BirA] = 0.5 μM; lower trace, [bio-5′-AMP] = 2.5 μM; upper trace, [biotin] = 2.5 μM. Inset, [BirA] = 2 μM and [biotin] = 10 μM. Measurements were performed in buffer A at 20.0 ± 0.1 °C.

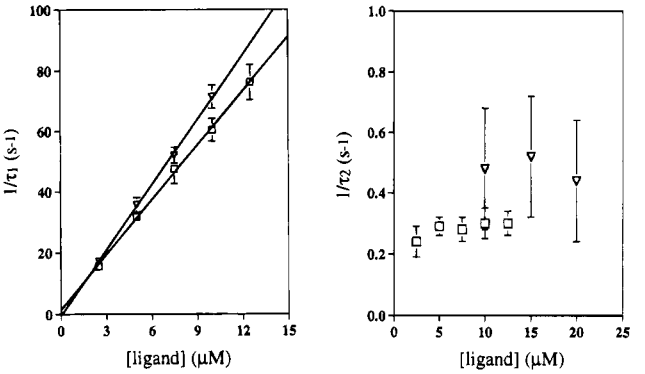

FIGURE 3: Dependence of the apparent rates of the (A) first ($1/\tau_1$) and (B) second ($1/\tau_2$) phases in the association of bio-5′-AMP (□) and biotin (▽) with BirA on ligand concentration.

double-exponential model:

$$F = A_1 \exp\left(\frac{1}{\tau_1} \times t\right) + A_2 \exp\left(\frac{1}{\tau_2} \times t\right) + C \qquad (1)$$

where F is the fluorescence intensity, A_1 and τ_1 and A_2 and τ_2 are the amplitudes and the relaxation times for the first and second phases, respectively, and C is the baseline fluorescence signal of the BirA−bio-5′-AMP complex.

The following general two-step model is consistent with the results of the kinetic data:

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

In the first step, which results in the major fraction of the fluorescence change, an enzyme−bio-5′-AMP encounter complex (BirA−bio-5′-AMP) is formed. The second step involves a relatively slow conformational change in the complex to its final form (*BirA−bio-5′-AMP). This model is supported by the ligand concentration dependence of the rates of the two phases. As predicted, the apparent rate of the first phase, $1/\tau_1$, is dependent on ligand concentration while that of the second, $1/\tau_2$, is independent of ligand concentration (Figure 3). For the general two-step reversible model shown in eq 2, the relationships between the apparent rates of the two experimentally observed phases and the

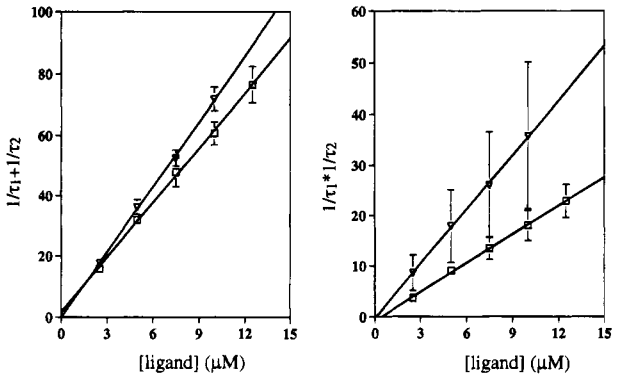

FIGURE 4: Dependence of (A) $1/\tau_1 + 1/\tau_2$ and (B) $1/\tau_1 \times 1/\tau_2$ on bio-5′-AMP (□) and biotin (▽) concentration.

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

ligand	k_1 (M^{-1} s^{-1})[a]	k_{-1} (s^{-1})	$(k_2 + k_{-2})$ (s^{-1})
bio-5′-AMP	5.9 (±0.8) × 10^6	1 ± 3	0.32 ± 0.09
biotin	7.1 (±0.8) × 10^6	0 ± 3	0.5 ± 0.2

[a] Parameters were estimated by weighted linear least-squares analysis of the kinetic data using eqs 3 and 4.

microscopic rate constants are as follows (Fersht, 1985):

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

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

All of the rate constants for the binding process can, in principle, be obtained by determining the dependence of the sum and product of the reciprocals of the time constants on ligand concentration. The results of this analysis are shown in Figure 4, and the values of the rate constants obtained from the analysis are shown in Table 1. Both k_1 and $(k_2 + k_{-2})$ for binding of bio-5′-AMP can be reliably estimated from the slopes of the dependence of the sum and product, respectively, of the reciprocal time constants, $1/\tau_1$ and $1/\tau_2$, on ligand concentration. k_{-1} was estimated from the intercept of the dependence of the sum of the apparent rate constants on bio-5′-AMP concentration. Since the magnitude of the intercept is small, the resulting error in k_{-1} is large.

Association of biotin with BirA was also measured by stopped-flow fluorescence and a representative trace is shown in Figure 2. The association is also well described by a double-exponential model. In contrast to bio-5′-AMP binding, which results in quenching of 40% of the intrinsic protein fluorescence signal, binding of biotin leads to quenching of only 15% of the signal. Consequently, the amplitude of the second phase in the process is very small. This small amplitude precluded analysis of the data obtained at a single BirA concentration using the formalism used for bio-5′-AMP binding above. Instead, data for the second phase were obtained at a protein concentration 4-fold higher than those used for measurement of the first rapid phase which can only be measured at the lower BirA concentration (Figure 2, inset). This approach is justified by the lack of dependence of the second phase on either protein or ligand concentration. The dependence of the apparent rates of the first and second phases on biotin concentration are shown in Figure 3. Microscopic rate constants obtained from analysis of the data using eqs 3 and 4 (Figure 4) are shown in Table 1. As

Table 2: Unimolecular Rate Constants for Dissociation of the *BirA−bio-5′-AMP and *BirA−Biotin Complexes

complex	k_{off} (s^{-1})
*BirA−bio-5′-AMP	2.7 (±0.3) × 10^{-4} [a]
*BirA−biotin	0.29 ± 0.01

[a] The rate of dissociation of the *BirA−bio-5′-AMP complex was obtained from the time course of BirA-catalyzed synthesis of bio-5′-AMP from the substrates biotin and ATP as described in Xu and Beckett (1994).

observed in binding of bio-5′-AMP, the uncertainty in the k_{-1} parameter for binding of biotin to BirA is large.

Measurement of Partitioning of the Intermediate Complexes. The values of k_{-1} for binding of both biotin and bio-5-AMP to BirA obtained from analysis of the association data above have large errors associated with them. More precise estimates of these parameters can be obtained from direct measurement of partitioning of the kinetic intermediates or collision complexes, BirA−ligand. As shown in eq 2, the collision complex can kinetically partition between dissociation to free ligand and protein and conversion to the final, tightly bound complex, *BirA−ligand. The rate of the dissociation process is governed by k_{-1} while that for conversion to *BirA−ligand is governed by k_2. The differences in the magnitudes of the time constants, τ_1 and τ_2, associated with the two kinetic phases in binding of both ligands to BirA are sufficiently large to allow direct measurement of partitioning of the collision complexes. Measurement of the partitioning will allow for more precise estimation of k_{-1}. Interpretation of the partitioning data requires knowledge of the unimolecular rate constants governing dissociation of the final complexes, *BirA−biotin and *BirA−bio-5′-AMP. The dissociation rate constant of the *BirA−bio-5′-AMP complex has been previously reported (Table 2) (Xu & Beckett, 1994). Stopped-flow fluorescence was used to measure the rate of dissociation of the BirA−biotin complex by rapid mixing of a 1:1 complex into a large excess of bio-5′-AMP. A trace of the resulting time-dependent change in the fluorescence intensity is shown in Figure 5. Since the intrinsic protein fluorescence of BirA complexed with bio-5′-AMP is lower than that of the protein complexed with biotin, a decrease in the fluorescence intensity is observed. A single-exponential model was used to analyze the data and excellent agreement between the best fit curve and the experimental data is observed. The best fit value for the dissociation rate constant for the complex is shown in Table 2.

Partitioning of the collision complexes was measured by stopped-flow fluorescence double mixing experiments. In order to sufficiently populate the intermediate complex, the protein and primary binding ligand, biotin or bio-5′-AMP, were first mixed in approximately a 1:1 molar ratio and allowed to react for approximately 10 half-lives of the first step in the two-step association process (Figure 2). The competitive ligand, bio-5′-AMP or biotin, was then rapidly mixed from the third syringe in large molar excess over the primary ligand, and the resulting time-dependent change in intrinsic protein fluorescence intensity was measured. Measurement of partitioning of the BirA-bio-5′-AMP intermediate is shown in Figure 6A. Curve a demonstrates the signal expected for binding of biotin to BirA. If 100% of the intermediate partitioned toward dissociation, the final fluorescence signal is expected to be equivalent that shown in curve a. Curve c represents the observed time-dependent

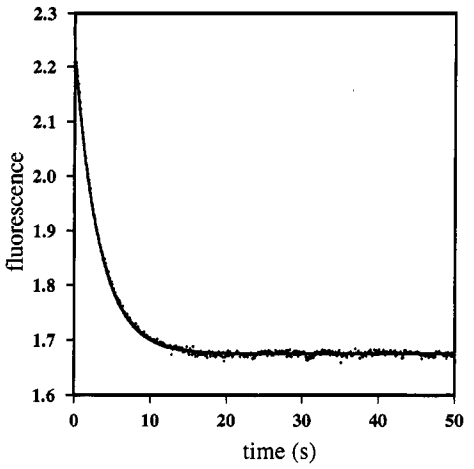

FIGURE 5: Stopped-flow fluorescence trace obtained for measurement of dissociation of the BirA−biotin complex. The measurement was made in buffer A. The pre-formed BirA−biotin complex ([BirA] = 2 μM, [biotin] = 2 μM) in buffer A was rapidly mixed with an equal volume of 200 μM bio-5′-AMP, and the resulting time-dependent decrease in fluorescence intensity was measured. The solid line represents the best fit of the data to a single-exponential model.

change in fluorescence observed when no competitive ligand is added from the third syringe. This trace is essentially what one would observe in the second phase in association of bio-5′-AMP with BirA (see Figure 2). Curve b indicates the observed time course when a large excess of the competing ligand, biotin, is mixed with the intermediate. Since addition of biotin to the intermediate results in an increase in the fluorescence intensity relative to that observed in curve c, we conclude that some partitioning of the collision complex toward dissociation does occur. The unimolecular rate constant for dissociation of the final complex is very small (Table 2). We do not know which rate constant, k_{-1} or k_{-2}, of the two that contribute to the overall dissociation process is smaller. If, however, the slow rate derives from very slow release of bio-5′-AMP from the intermediate complex, no dissociation of the intermediate should occur in the time frame of measurement shown in Figure 6A. The observed increase in fluorescence intensity provides clear evidence for some dissociation of the intermediate to free ligand and protein and, therefore, indicates that the slower step in overall dissocation of the complex is that governed by k_{-2}. The partitioning data could not be analyzed directly to obtain a value for k_{-1} because of the high degree of correlation among the parameters in the analysis. The data can, however, be simulated using a simple double-exponential model governed by the two rate constants, k_{-1} and k_2. Based on the known value of ($k_2 + k_{-2}$) in Table 1 and the conclusion that the step governed by the constant k_{-2} is the slower step in the overall dissociation of the *BirA−bio-5′-AMP complex, k_2 was fixed at 0.3 s^{-1} for the simulations. The value of the amplitude for each kinetic phase used in the simulations was obtained from the experimentally measured values of the fluorescence of the intermediate, the BirA−biotin, and the BirA−bio-5′-AMP complexes. The simulations indicate that the final value of the fluorescence intensity observed in the partitioning measurement is consistent with a value of k_{-1} of approximately 0.2 s^{-1} (Figure 6A). Moreover, the curve reflects the combination of two processes, one with an increase in fluorescence intensity associated with it and the other with a decrease in intensity. At some combination of the rate constants, k_{-1} and k_2, the observed fluorescence intensity is expected to be independent of time. This corresponds to a value of k_{-1} of approximately 0.2 s^{-1}.

Partitioning of the BirA−biotin intermediate complex was measured by a method analogous to that used for the BirA−

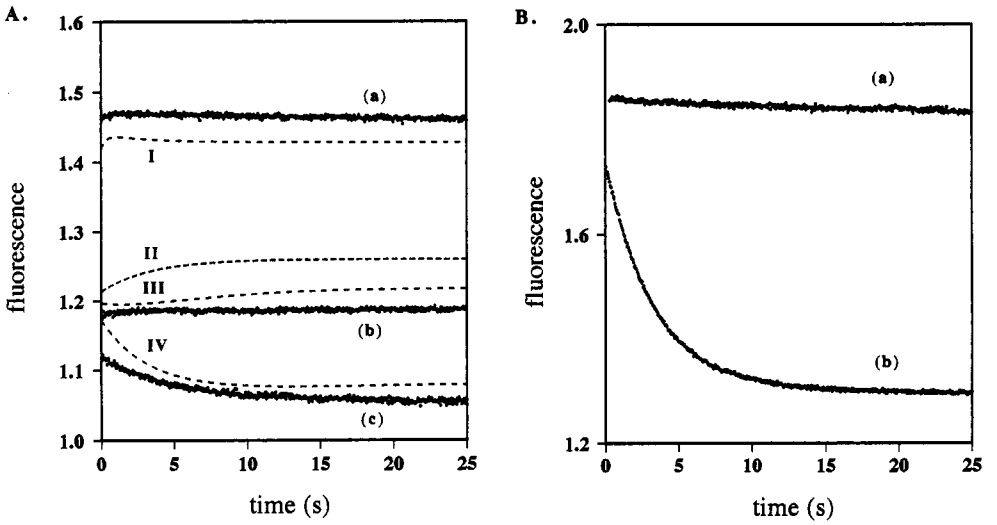

FIGURE 6: (A) Stopped-flow fluorescence measurement of partitioning of the BirA−bio-5′-AMP intermediate complex. $[BirA]_{final}$ = 0.6 μM in all kinetic traces. (a) Syringe A, BirA; syringe B, buffer A; syringe C, biotin, $[biotin]_{final}$ = 170 μM. (b) Syringe A, BirA; syringe B, bio-5′-AMP; syringe C, biotin, $[bio\text{-}5'\text{-}AMP]_{final}$ = 1.2 μM, $[biotin]_{final}$ = 170 μM. (c) Syringe A, BirA; syringe B,bio-5′-AMP; syringe C, Buffer A, $[bio\text{-}5'\text{-}AMP]_{final}$ = 1.2 μM. In all measurements the contents of syringes A and B were mixed and allowed to react for 0.8 s (approximately nine half-lives of the first kinetic process) and then mixed with the contents of syringe C. Data collection was initiated following the second mixing step. All experimental curves are the average of 5−10 determinations. Curves I−IV represent simulations of the partitioning data assuming values of k_{-1} ranging from 3.0 s^{-1} to 0.03 s^{-1}. The curves were simulated using the following equation: $F = f_1A_1 \exp(-k_{-1}t) + f_2A_2 \exp(-k_2t)$ where F is fluorescence, t is time in seconds, and k_2 = 0.3 s^{-1}, A_1 = −0.27, and A_2 = 0.15 in all curves. f_1 and f_2 represent the fractional contribution of each single-exponential process to the total fluorescence change. Values of k_{-1} used in the simulated curves are as follows: **I**, 3.0 s^{-1}; **II**, 0.3 s^{-1}; **III**, 0.2 s^{-1}; and **IV**, 0.03 s^{-1}. (B) Partitioning of the BirA−biotin intermediate complex. $[BirA]_{final}$ = 0.8 μM in all kinetic traces. (a) Syringe A, BirA; syringe B, biotin; syringe C, buffer A, $[biotin]_{final}$ = 1.2 μM. (b) Syringe A, BirA; syringe B, biotin; syringe C, bio-5′-AMP, $[biotin]_{final}$ = 1.2 μM, $[bio\text{-}5'\text{-}AMP]_{final}$ = 120 μM. In both measurements the contents of syringes A and B were allowed to react for 0.8 s (approximately nine half-lives of the first kinetic process) and then mixed with the contents of syringe C. Data collection was initiated following the second mixing step.

bio-5′-AMP complex. The measured overall rate constant for dissociation of the *BirA−biotin complex of 0.3 s^{-1} places a lower limit on the value of the constant, k_{-1}, governing dissociation of the intermediate to free protein and ligand. Addition of a large molar excess of bio-5′-AMP to the BirA−biotin intermediate complex results in a time-dependent decrease in the fluorescence signal (Figure 6B). Since the amplitude of the fluorescence change associated with the conversion of the intermediate complex to its final form is very small, it does not contribute to the overall observed change in fluorescence. The observed change, therefore, reflects the sum of the population of intermediate complexes that immediately dissociates upon mixing with excess bio-5′-AMP and the population that goes on to final form and then dissociates. If the value of k_{-1} were significantly greater than the measured value of k_{off}, two phases would be observed in the partitioning experiment. The decrease in fluorescence intensity is, however, well described by a single-exponential model, and nonlinear least-squares analysis of the data yields a rate constant for the process of 0.3 s^{-1}, a value identical to that measured for the overall dissociation of the BirA−biotin complex. We, therefore, conclude that k_{-1} is indistinguishable from k_{off} for binding of BirA to biotin.

The Apparent Second-Order Association and First-Order Dissociation Rate Constants for the BirA−Ligand Interactions. The apparent second-order rate constant, k_{on}, for association of BirA with biotin or bio-5′-AMP in the two-step binding reactions are obtained from the microscopic rate constants for the process shown in eq 2 from the following relationship:

$$k_{on} = \frac{k_1 k_2}{k_{-1} + k_2} \tag{5}$$

Based on the values of the microscopic rate constants, $k_2/(k_{-1} + k_2)$ is approximately 0.5 for binding of both ligands and $k_{on} \approx 0.5k_1$. The relationship between the first-order rate constants, k_{off}, governing dissociation of the complexes and the microscopic rate contants is

$$k_{off} = \frac{k_{-2} k_{-1}}{k_{-1} + k_2}$$

Again, since $k_{-1}/(k_{-1} + k_2)$ is approximately equal to 0.5 for both complexes, $k_{off} \approx 0.5k_{-2}$.

Thermodynamics of Binding of BirA to Biotin and Bio-5′-AMP. Direct measurement of the dissociation equilibrium constant for the BirA−bio-5′-AMP interaction using equilibrium titration techniques was not successful because the stability of the complex is very high. Only the stoichiometry of the binding process, which is 1:1, could be obtained (Abbott & Beckett, 1993). Direct equilibrium measurements of binding of biotin to BirA have been made by monitoring the change in the intrinsic protein fluorescence signal as a function of biotin concentration. Results of analyses of several data sets indicate that the equilibrium dissociation constant for the interaction is in the range of 3×10^{-8} M to 1×10^{-7} M (D.B., unpublished data). The uncertainty in the number is due to the fact that the measurements were, by necessity, performed at a protein concentration that is close to the stoichiometric limit of binding (Weber, 1992). The kinetic results reported in this work can be used to obtain equilibrium dissociation constants for binding of both ligands to BirA using the following relationship:

$$K_D = \frac{k_{off}}{k_{on}} \tag{6}$$

The expressions for k_{off} and k_{on} in terms of the microscopic rate contants have been discussed above, and the resulting expression for the equilibrium constant is $K_D = k_{-2}/k_1$. The equilibrium dissociation constants and Gibbs free energies of binding for the BirA−ligand interactions are given in Table 3. The value calculated from the kinetic data agrees well with that obtained from direct equilibrium titrations of BirA with biotin. Both the complexes of biotin and bio-5′-AMP with BirA are relatively stable in the conditions used for the measurements. Addition of the AMP moiety to biotin leads to an increase in stability of approximately −4 kcal/mol.

Proteolysis. Results of kinetic measurements discussed above indicate that association of both biotin and bio-5′-AMP with BirA is at least a two-step process. In the first step a collision complex between protein and ligand is formed, and in the second a conformational transition in the enzyme occurs. The kinetic data indicate, moreover, that there are two distinct ligand bound states of BirA. Proteolysis was used as an additional probe of the conformation of BirA in its complexes with biotin and bio-5′-AMP. Partial proteolysis of BirA with the relatively nonspecific endoprotease, subtilisin, yields the two products shown in Figure 7A. The sizes of these products are estimated by SDS−PAGE to be approximately 25 and 10 kDa. The products of digestion were electroblotted onto PVDF membrane and subjected to automated Edman degradation to determine the point of cleavage. Results of the N-terminal sequencing indicate that the cleavage occurs at the peptide bond that links amino acid residues 217 and 218 (Howard et al., 1985). The three-dimensional structure of BirA determined by X-ray crystallographic techniques (Wilson et al., 1992) indicates that the peptide bond at which subtilisin-catalyzed cleavage occurs is found in one of four "unstructured" loops.

Kinetics of Proteolysis. The time course of subtilisin-catalyzed proteolysis of BirA was measured in the absence and presence of ligands and the amount of intact BirA present at each time point was quantitated by densitometry of a Coomassie-stained gel. The cleavage reaction was treated as a simple first-order process in order to obtain apparent rates (slope = $-k$) of proteolysis, and graphical representations of the analysis are shown in Figure 7B. The rates of cleavage indicate that binding of either biotin or bio-5′-AMP results in protection of the loop from cleavage by subtilisin. Binding of biotin to BirA results in roughly a 2-fold decrease in the rate of proteolysis relative to the unliganded protein while binding of bio-5′-AMP results in approximately a 10-fold decrease.

DISCUSSION

The Kinetics of Association of BirA with Biotin and Bio-5′-AMP. The results of stopped-flow fluorescence measurements of the association of BirA with biotin and bio-5′-AMP reported in this work indicate that both binding processes are complex. They consist of two phases which we have interpreted as initial rapid formation of a collision complex between the protein and ligand, followed by slow isomerization of the complex to a more stable form. We have

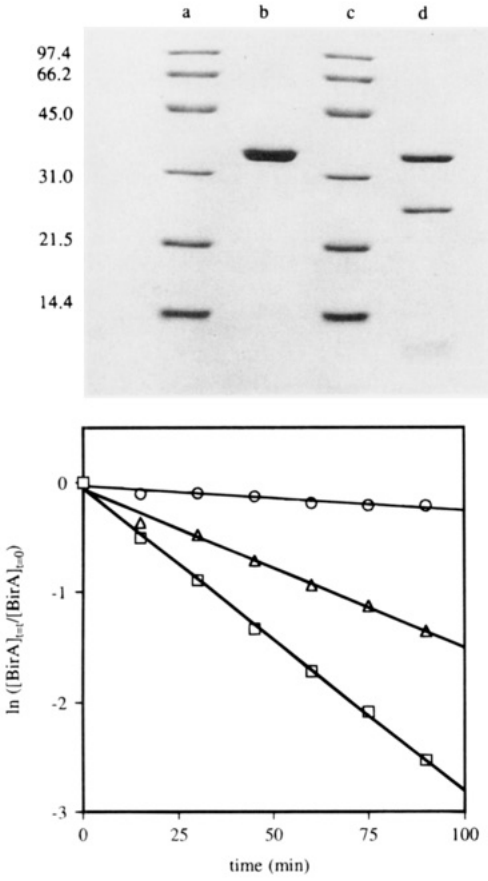

FIGURE 7: (A, top) Proteolytic digestion pattern obtained from treatment of BirA with subtilisin. (Lanes a and c) Molecular weight markers, (lane b) intact BirA, (lane d) BirA subjected to subtilisin digestion. (B, bottom) Kinetic time courses of subtilisin-catalyzed cleavage of 5 μM BirA (□), 5 μM BirA + 100 μM biotin (△), and 5 μM BirA + 100 μM bio-5′-AMP (○). All measurements were performed in buffer A.

Table 3: Equilibrium Dissociation Constants and Gibbs Free Energies for Binding of Biotin and Bio-5′-AMP to BirA

complex	K_D (M)[a]	ΔG (kcal/mol)
BirA−bio-5′-AMP	5 (±2) × 10^{-11}	−13.9 ± 0.3
BirA−biotin	4.1 (±0.5) × 10^{-8}	−9.9 ± 0.3

[a] Equilibrium dissociation constants were calculated from the measured bimolecular association rate constants for binding of the ligands to BirA, k_1 (Table 1), and unimolecular rate constants governing dissociation of the complexes, k_{off} (Table 2).

previously reported that binding of bio-5′-AMP to BirA is well described by a single-exponential model (Xu & Beckett, 1994). In this work, extension of the time scale of the measurements enabled observation of the second slow step in the reaction. For both ligands, the large dependence of the time constant of the first phase on ligand concentration supports the contention that it reflects formation of a collision complex. The value of k_1, the bimolecular rate constant for formation of the collision complex, obtained from analysis of the kinetic data ranges from 6 × 10^6 M^{-1} s^{-1} to 7 × 10^6 M^{-1} s^{-1} for the two ligands. This value is considerably lower than values expected for a diffusion controlled process. However, such low values have been reported in other systems (Simopoulos & Jencks, 1994) and may reflect, for example, the requirement for collision of the ligand with the protein to occur in a particular orientation for productive complex formation.

We have interpreted the second slow phase in association of BirA with the two ligands as reflecting the isomerization of the complex to a more stable form. The lack of dependence of the time constant for this second phase on ligand concentration is consistent with this interpretation. It has previously been reported that addition of either bio-5′-AMP or biotin to crystals of apoBirA results in cracking of the crystals (Wilson et al., 1992), an observation consistent with the occurence of a structural change in the protein upon binding of either ligand. The results presented in this work indicate that the particular conformation of the protein reflects which ligand is bound to it. The amplitude of the second kinetic phase is considerably larger for bio-5′-AMP binding than for binding of biotin. Moreover, the kinetics of the change in conformation are quantitatively different for the two ligands. The value of k_{-2}, the first-order rate constant governing isomerization of *BirA−ligand to BirA−ligand, is approximately 3 orders of magnitude slower for the bio-5′-AMP-bound protein than for the biotin-bound form. Moreover, the rates of subtilisin-catalyzed proteolytic digestion of the protein bound to the two ligands are distinct. The different rates of proteolysis reflect differences in the accessibility of the the peptide bond between residues 217 and 218 in the two liganded states of the enzyme.

Functional Significance of the Conformational States of BirA. The conformational change associated with biotin binding may be relevant to the mechanism of catalysis of bio-5′-AMP synthesis. The estimated intracellular concentration of biotin is in the range of 10 nM (Pai, 1972; Prakash & Eisenberg, 1974) while that of ATP is in the millimolar range (Kornberg & Baker, 1992). Thus, biotin is the limiting substrate in the enzymatic reaction. Moreover, ATP appears to bind only very weakly to BirA in the absence of biotin (Y.X. and D.B., unpublished results). Binding of the two substrates to BirA to form the ternary enzyme−substrate complex may, therefore, occur in an ordered fashion with biotin binding first. The structural transition that occurs upon binding of biotin to BirA may be required to allow for tight binding of the second substrate, ATP. No additional change in the conformation of the enzyme apparently occurs until bio-5′-AMP is synthesized.

Site-specific binding of BirA to the biotin operator is allosterically regulated by bio-5′-AMP. In the absence of the effector, no binding of BirA to bioO is observed. Clearly the conformation of BirA in its complex with bio-5′-AMP is the active conformation in sequence-specific DNA binding. Although the detailed mechanism of the allosteric activation is not known, we have previously shown that the mechanism of binding of BirA to the biotin operator involves cooperative association of two holorepressor monomers with the two half-sites of the operator (Abbott & Beckett, 1993). The cooperativity may, at the structural level, involve formation of a protein−protein interface. Results of sedimentation equilibrium studies indicate that binding of bio-5′-AMP to BirA is positively linked to dimerization of the protein (Eisenstein and D.B., unpublished observations). Allosteric activation of the site-specific DNA binding function of BirA by bio-5′-AMP is related to the dimerization function and formation of stable BirA dimers requires that the protein be in the appropriate conformation.

The Affinities of BirA for Biotin and Bio-5′-AMP. Both biotin and bio-5′-AMP bind with high affinity to BirA in the conditions employed in this work. The Gibbs free energy for biotin binding is approximately −10 kcal/mol, while that

for bio-5′-AMP binding is −14 kcal/mol. Addition of the AMP moiety to biotin thus results in an increase in the protein−ligand stability of −4 kcal/mol. These values for the Gibbs free energies of binding are calculated using equilibrium dissociation constants obtained from the kinetic data. Examination of the detailed kinetics of binding of both ligands indicates that the increased stability of the BirA−bio-5′-AMP complex is reflected primarily in a markedly decreased rate of dissociation of the ligand−protein complex. The overall rate constant governing dissociation of *BirA−bio-5′-AMP to free ligand and protein is 0.00027 s^{-1}. The estimated value of k_{-1}, the rate constant governing dissociation of the intermediate complex, BirA−bio-5′-AMP, determined from the partitioning experiments is 0.2 s^{-1}. The slow overall dissociation rate, therefore, reflects the very slow isomerization of the final complex, *BirA−bio-5′-AMP, to the intermediate complex, BirA−bio-5′-AMP. The overall dissociation constant, k_{off}, governing dissociation of the *BirA−biotin complex is 0.3 s^{-1}, and the value of k_{-1} estimated from the partitioning experiments is identical to k_{off}. k_{-2} is also approximately 0.3 s^{-1} for dissociation *BirA−biotin. The difference in affinity of the two ligands for BirA, therefore, primarily reflects distinct rate parameters governing the conformational change in the two protein−ligand complexes.

The equilibrium dissociation constant for the BirA−biotin interaction is approximately 40 nM, which is in the same range as the intracellular biotin concentration (Pai, 1972; Prakash & Eisenberg, 1974). The mechanism of BirA-catalyzed synthesis of bio-5′-AMP may involve ordered binding of the two substrates with biotin binding first. The high affinity of BirA for biotin and its apparently low affinity for ATP (Y.X. and D.B., unpublished results) are consistent with this type of mechanism.

Bio-5′-AMP binds with significantly higher affinity to BirA than does biotin. The complex of BirA with bio-5′-AMP is required for two functions, sequence-specific binding to bioO and binding to BCCP, followed by transfer of biotin. The high affinity of BirA for the effector may be rationalized in terms of these two functions. Bio-5′-AMP is a mixed anhydride and can undergo hydrolysis in aqueous solution. If the adenylate were readily released into the cytoplasm, a significant fraction of the compound would be hydrolyzed and, consequently, wasted instead of being utilized productively in the biotin transfer reaction or as a corepressor in DNA binding. The second reason for the unusually high stability of the BirA−bio-5′-AMP complex may be related to its transcriptional regulatory role. Bio-5′-AMP allosterically activates BirA for binding to bioO, and formation of this sequence-specific DNA−protein complex results in repression of transcription of the biotin biosynthetic operon. In its adenylated form, biotin serves as a feedback inhibitor of its own biosynthesis. If the affinity of bio-5′-AMP for BirA were low, a significant fraction of the repressor would exist in the free or biotin bound forms, neither of which binds tightly to bioO. The switch in BirA function from biotin ligase to DNA-binding protein occurs once the metabolic requirement for biotin is satisfied, and the supply of the apoBCCP is completely converted to the holo form. The high stability of the BirA−bio-5′-AMP complex ensures that the repressor is always in the form that is active for binding tightly to bioO, thereby guaranteeing stringent linkage between the metabolic requirement for and biosynthesis of biotin.

Summary. The 35.3 kDa repressor of biotin biosynthesis is an example of the efficient utilization of a protein sequence of limited size to carry out multiple functions. Its 321 amino acid residues code for catalysis of two chemical reactions that require binding of multiple small molecules as well as a second heterologous protein. BirA also binds sequence-specifically to DNA in a reaction that is linked to both small molecule effector binding and protein assembly. The occurence of conformational transitions in a protein can provide a mechanism for obtaining multiple functions from a single coding sequence and, moreover, regulating function. The kinetic studies presented in this work provide evidence for the existence of at least three conformational states of BirA. In addition, the results indicate that it is the difference in the kinetics of the conformational transition that provides the basis for the large difference in thermodynamic stability of two liganded forms of BirA. This difference in thermodynamic stability is critical for maintaining BirA in an "activated" state in the cell. The results of partial proteolytic digestion provide additional evidence for distinct conformational states of the protein. Future high-resolution X-ray crystallographic studies of the ligand bound states of BirA will further increase our understanding of the structural basis of regulation of function in this unique protein.

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