Kinetics of Biotinyl-5′-adenylate Synthesis Catalyzed by the *Escherichia coli* Repressor of Biotin Biosynthesis and the Stability of the Enzyme–Product Complex†

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ABSTRACT: The *Escherichia coli* repressor of biotin biosynthesis is both a biotin ligase and the repressor of transcriptional initiation at the biotin biosynthetic operon. The small molecule, biotinyl-5′-adenylate (bio-5′-AMP), is the intermediate in the biotin ligation reaction *and* the positive allosteric effector for sequence-specific DNA binding by BirA. Synthesis of the adenylate from the substrates biotin and ATP is catalyzed by BirA. Although BirA and other biotin holoenzyme synthetases have been the subject of biochemical studies, no direct measurements of the bio-5′-AMP synthesis reaction have been reported. No information relating to the mechanism and kinetic parameters governing adenylate synthesis is available. In addition to this lack of kinetic information, the thermodynamic stability of the BirA–bio-5′-AMP complex is not known. Since the BirA–adenylate complex plays a pivotal role in the biotin regulatory system, both the kinetic and thermodynamic information are essential to a quantitative understanding of the system. We have developed a method for measuring the time course of bio-5′-AMP synthesis. The results of these measurements indicate that the time course is characterized by an initial burst followed by a slow linear phase. The burst corresponds to the rapid synthesis of 1 mol of product per mole of enzyme, and the rate of the slow linear phase is limited by the release of product from the enzyme. The data have been analyzed to obtain the dissociation rate constant of 3.9×10^{-4} s^{-1} for the BirA–bio-5′-AMP complex. Stopped-flow fluorescence measurements have been utilized to obtain the bimolecular association rate constant of 4.9×10^{6} M^{-1} s^{-1} for complex formation. The equilibrium dissociation constant for the BirA–bio-5′-AMP interaction of 7.9×10^{-11} M has been determined from the two kinetic parameters, and the resulting Gibbs free energy for complex formation is −13.5 kcal/mol.

Biotin biosynthesis and retention in *Escherichia coli* are regulated via the set of macromolecular interactions outlined in Figure 1. Biotin is retained in the cell in two protein-bound forms: as a covalent adduct of the biotin acceptor subunit of cellular carboxylases or in a noncovalent complex of the adenylated form bound to the repressor of biotin biosynthesis (BirA). The major biotin-dependent carboxylase in *E. coli* is the acetyl-CoA carboxylase, in which the biotinated subunit is the biotin carboxyl carrier protein (BCCP). The results of examination of intracellular biotin pools in *E. coli* indicate that the fraction of intracellular biotin in the bound form, both noncovalent and covalent forms, can be as high as 80% (Pai, 1972; Prakash & Eisenberg, 1974). The multifunctional protein, BirA, is critical for biotin retention since it catalyzes the covalent ligation of biotin to BCCP. This reaction occurs via an acyl activation mechanism involving the formation of the enzyme-bound adenylate, bio-5′-AMP (Prakash & Eisenberg, 1979) (reaction I in Figure 1). In addition to functioning as the intermediate in the biotin ligation reaction, the BirA–bio-5′-AMP complex regulates biotin biosynthesis via its sequence-specific binding to the biotin operator sequence. Formation of this protein–DNA complex results in the repression of transcription initiation at the biotin biosynthetic operon by blocking binding of the RNA polymerase to the two promoters, Pa and Pb (Otsuka & Abelson, 1978). In this system, bio-5′-AMP functions as both the activated intermediate in the biotin-transfer reaction and as a positive allosteric effector for the binding of BirA to the biotin operator sequence (Prakash & Eisenberg, 1979). The switch in BirA function from biotin ligase to transcriptional repressor is regulated by the intracellular biotin concentration and by the intracellular apo-BCCP pool (Cronan, 1989).

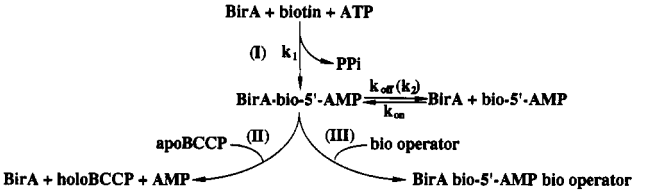

FIGURE 1: Schematic representation of the interactions that contribute to regulation of biotin biosynthesis and retention in *E. coli*. BirA's functions include (I) catalysis of the synthesis of bio-5′-AMP from biotin and ATP, (II) transfer of biotin from the adenylate to BCCP, and (III) sequence-specific binding to the biotin operator sequence. k_1 is the rate constant for the chemistry of formation of bio-5′-AMP from the substrates biotin and ATP, k_{off} (k_2) is the first-order rate constant governing the release of bio-5′-AMP from BirA, and k_{on} is the bimolecular association constant for the formation of the BirA–bio-5′-AMP complex.

Since formation of the BirA–bio-5′-AMP complex is a prerequisite for both the ligase and DNA binding functions of BirA, a quantitative understanding of either function, and the switch from one to the other, requires the determination of the thermodynamics of the BirA–adenylate interaction. Previously published studies on BirA indicate that bio-5′-AMP is tightly bound to BirA. For example, the BirA–bio-5′-AMP complex is stable to gel filtration chromatography (Prakash & Eisenberg, 1979). Attempts to measure the equilibrium constant for the BirA–bio-5′-AMP interaction by fluorescence titration were unsuccessful because the

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equilibrium dissociation constant appears to be in the subnanomolar concentration range (Nenortas, E., Xu, Y., Abraham, T., & Beckett, D., submitted for publication). This apparent high affinity of the adenylate for BirA renders direct determination of the stability of the complex by equilibrium techniques difficult, if not impossible. A kinetic approach to determining the thermodynamic stability of the BirA–bio-5′-AMP complex may, however, prove fruitful.

BirA is one of a class of enzymes termed the biotin holoenzyme synthetases, all of which catalyze the covalent attachment of biotin to enzymes that carry out biotin-dependent transcarboxylation reactions. Several of these ligases have been purified and subjected to biochemical studies. In the majority of cases, the activities of the enzyme have been determined indirectly by monitoring the activity of the holotranscarboxylase, the ligation reaction product (Lane *et.al.*, 1964; Siegel *et al.*, 1965; Cazzulo *et al.*, 1970, 1971). One case of direct measurement of the kinetics of incorporation of biotin into the biotin acceptor protein subunit has been reported (Shenoy & Wood, 1988). Although all of the biotin holoenzyme synthetases function via the formation of an adenylated intermediate, no quantitative measurement of the reaction involving the synthesis of bio-5′-AMP (reaction I in Figure 1) from the substrates biotin and ATP has been reported for any of these enzymes. Measurement of this half-reaction can provide kinetic parameters for the process and information about the enzyme–adenylate interaction that may be relevant to determining the thermodynamic stability of that complex.

In this article, we present results of direct measurement of the time course of BirA-catalyzed synthesis of bio-5′-AMP. The time course is characterized by an initial exponential burst followed by a slow linear phase. The initial burst corresponds to the synthesis of 1 mol of the adenylate per mole of enzyme. The linear phase reflects the slow release of product from the enzyme. We have utilized measurements of the rapid exponential phase and the slow linear phase to estimate the initial rate of bio-5′-AMP synthesis at saturating biotin concentration and the first-order rate of release of bio-5′-AMP from the complex. The results of these latter measurements indicate that the half-life of the complex under the conditions employed is >30 min. The bimolecular association of BirA with bio-5′-AMP has been measured directly by stopped-flow fluorescence techniques. The rate constant obtained from these measurements indicates that the association process is significantly slower than the diffusion-controlled limit, which may reflect the occurrence of a conformational change in the protein upon binding of the ligand. The directly measured bimolecular association constant governing formation of the BirA–bio-5′-AMP complex and the dissociation rate constant obtained from measurements of the linear phase of the time course of adenylate synthesis have been combined to determine an equilibrium dissociation constant for the BirA–bio-5′-AMP interaction. The resulting equilibrium constant, approximately 10^{-10} M, indicates that the BirA–adenylate complex is very stable. The unusually high kinetic stability of the BirA–bio-5′-AMP complex indicated by these measurements suggests that *in vivo* BirA may always be activated either for interaction with BCCP and the subsequent transfer of biotin or for binding to the biotin operator. We suggest that this allows for stringent linkage between the two functions of the BirA–adenylate complex.

MATERIALS AND METHODS

Chemicals and Biochemicals. All chemicals used in the preparation of buffers and in the mobile phase for thin-layer chromatography (TLC) were reagent grade. ATP and *d*-biotin were purchased from Sigma. [α-^{32}P]ATP (>400 Ci/mmol, EtOH/H_2O = 1/1) was purchased from Amersham. Inorganic pyrophosphatase (200 units/mg) was obtained from Boehringer Mannheim. Bio-5′-AMP was synthesized using a modification of the method outlined in Lane *et al.* (1965) (Abbott & Beckett, 1993). BirA was purified according to the method described in Abbott and Beckett (1993).

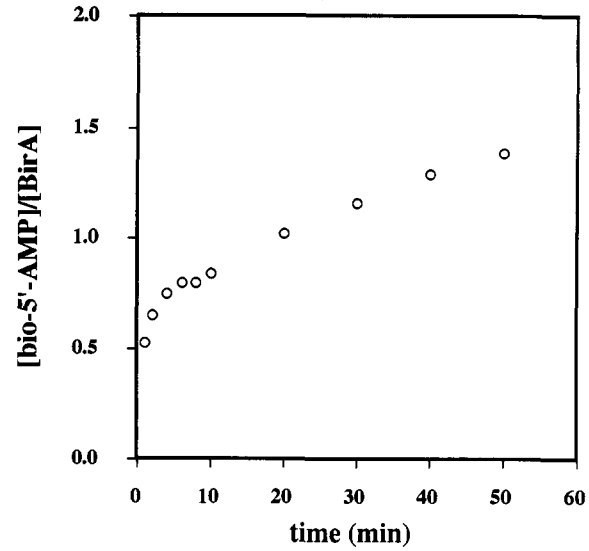

FIGURE 2: Time course of BirA-catalyzed synthesis of bio-5′-AMP measured by the quantitation of [^{32}P]bio-5′-AMP and [^{32}P]ATP resolved by thin-layer chromatography. [ATP] = 50 μM, [biotin] = 106 μM, and [BirA] = 5 μM. The reaction was carried out in buffer A at 20 °C.

Measurement of the 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, ATP, and biotin in buffer A (10 mM Tris-HCl, 200 mM KCl, and 2.5 mM $MgCl_2$, pH 7.50 ± 0.01 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 the 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. One microliter 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 v/v/v). Quantitation of 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. The 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 (Figure 2).

Steady-State Fluorescence Spectra. Fluorescence spectra were measured using an SLM 48000 spectrafluorimeter. The excitation wavelength was 295 nm, and emission was monitored

from 310 to 450 nm. The temperature was maintained at 20 °C using a circulating water bath. Excitation and emission slit widths were both set at 4 nm. The data were collected in the ratio mode using rhodamine B as the quantum counter. All spectra were corrected for the contribution of buffer A and for dilution.

Initial Rates of Bio-5′-AMP Synthesis. The intrinsic protein fluorescence spectra of BirA complexed to biotin and bio-5′-AMP are distinct, and the different spectral properties were utilized to measure the initial rate of BirA-catalyzed synthesis of bio-5′-AMP. Measurements were performed using a KinTek stopped-flow instrument. All BirA, substrates, and buffer preparations used in the measurements were filtered through 0.45-μm Acrodisc PTFE filters (Gelman Sciences). Of the three total sample syringes, two were filled with 3 μM BirA, 300 μM biotin, and 3 units/mL pyrophosphatase in buffer A. The third syringe contained ATP, the concentration of which was varied over a broad range. The reaction was initiated by rapid mixing of equal volumes (33 μL) of the solutions present in the three syringes. Reaction time courses were monitored by fluorescence using an excitation wavelength of 300 nm and then measuring fluorescence emission above 340 nm using a cutoff filter. Data were collected for 100 s for each shot. The temperature was maintained at 20.0 ± 0.1 °C using a circulating water bath.

Measurement of the Bimolecular Association Rate Constant Governing Formation of the BirA–Bio-5′-AMP Complex. The bimolecular rate of association of BirA with bio-5′-AMP was obtained by monitoring the change in the intrinsic protein fluorescence following combination of the protein and ligand. All measurements were made using a KinTek stopped-flow instrument. Two of the instrument syringes contained 1.5 μM BirA and the third contained bio-5′-AMP, the concentration of which was varied in separate measurements. Reactions were initiated by rapid mixing of 33-μL volumes from each of the three syringes. The excitation wavelength was set at 295 nm, and fluorescence emission wavelength was monitored above 340 nm. Data were collected for 0.5 s for each shot. The temperature was maintained at 20.0 ± 0.1 °C using a circulating water bath.

Data Analysis. All kinetic data were analyzed using a modified Gauss–Newton algorithm (Straume *et al.*, 1991). Details of the models used in the data analysis are described in Results. Single-exponential models were found to apply to measurements of the initial rates of bio-5′-AMP synthesis and the bimolecular association of BirA with bio-5′-AMP. A simple linear model was used to analyze the slow phase of the time course of bio-5′-AMP synthesis. Propagation of errors in the calculation of equilibrium parameters from the rate parameters was performed according to Bevington (1969).

RESULTS

Kinetics of BirA-Catalyzed Synthesis of Bio-5′-AMP. We have developed a method for monitoring the time course of BirA-catalyzed synthesis of bio-5′-AMP 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 using the Molecular Dynamics phosphorimaging densitometer. The broad dynamic range of the phosphorimaging system renders it particularly well-suited for quantitation of the bio-5′-AMP synthesis reaction, since the maximum amount of product synthesized during the time course, even in conditions of a large excess of the substrate ATP, is on the same order of magnitude as the enzyme concentration. The TLC spot corresponding to bio-5′-AMP was identified as such by its comigration with chemically synthesized bio-5′-AMP. Moreover, consistent with previous reports, the synthesis of bio-5′-AMP was shown to depend on the presence of biotin, ATP, and Mg^{2+} (Lane *et al.*, 1964; Cazzulo *et al.*, 1970; Shenoy & Wood, 1988). Bio-5′-AMP synthesis was also shown to be inhibited in the presence of an excess of the unlabeled, chemically synthesized bio-5′-AMP.

A time course of bio-5′-AMP synthesis is shown in Figure 2. All reactions involving BirA-catalyzed synthesis of bio-5′-AMP discussed in this work were carried out in the presence of inorganic pyrophosphatase. In the absence of this secondary enzyme, equilibrium levels of product were low since PP_i can combine with the adenylate in the reverse reaction. The inclusion of inorganic pyrophosphatase, the activity of which is high in the cell (Kornberg & Baker, 1991), serves to render the reaction irreversible. The presence of this secondary enzyme has no effect on product analysis since the amount of bio-5′-AMP synthesized is directly quantitated, rather than the amount of pyrophosphate or inorganic phosphate. The time course of bio-5′-AMP synthesis is characterized by a rapid exponential burst followed by a slow linear phase. The following chemical equation can adequately describe this process:

$$\text{BirA} + \text{biotin} + \text{ATP} \xrightarrow{k_1} \text{BirA–bio-5′-AMP} \xrightarrow{k_2} \text{BirA} + \text{bio-5′-AMP} \quad (1)$$

where the first step in the reaction, involving the synthesis of bio-5′-AMP, corresponds to the rapid burst in the time course, and the second step, which is rate-limited by the release of product from BirA, corresponds to the slow linear phase. k_1 is the apparent rate constant for the enzyme-catalyzed synthesis of bio-5′-AMP, and k_2 is the rate constant governing the release of adenylate from the BirA–adenylate complex. On the basis of this reaction scheme, the concentration of bio-5′-AMP synthesized at any time during the course of the reaction is expressed as follows (Fersht, 1985): where k_1 is the

$$[\text{bio-5′-AMP}]_t = n[\text{BirA}]_0\left(\frac{k_1}{k_1 + k_2}\right)\left(\frac{k_1}{k_1 + k_2} - \frac{k_1 e^{-(k_1+k_2)t}}{k_1 + k_2} + k_2 t\right) \quad (2)$$

rate constant for the synthesis of bio-5′-AMP, $[\text{BirA}]_0$ is the total concentration of the enzyme, and k_2 is the first-order rate constant for dissociation of the BirA–bio-5′-AMP complex. If the reaction conditions are such that [biotin] and [ATP] are much greater than $[\text{BirA}]_0$, and if $k_1 \gg k_2$ and $t \gg (k_1 + k_2)^{-1}$, then eq 2 reduces to the following linear form:

$$[\text{bio-5′-AMP}]_t = n[\text{BirA}]_0 + n[\text{BirA}]_0 k_2 t \quad (3)$$

where n corresponds to the stoichiometry of the burst complex. The slope of the line, $n[\text{BirA}]_0 k_2$, provides information about the rate of release of bio-5′-AMP from the BirA–bio-5′-AMP complex.

Provided that the enzymatic reactions are carried out according to the stipulations of the simplification of eq 3, there is a predicted dependence of the slopes of the linear phase of the enzymatic time course on enzyme concentration. Time courses were, therefore, performed at BirA concentrations ranging from 1 to 5 μM. Experiments were limited to this concentration range because at concentrations lower than 1 μM, the amount of radioactivity incorporated into product

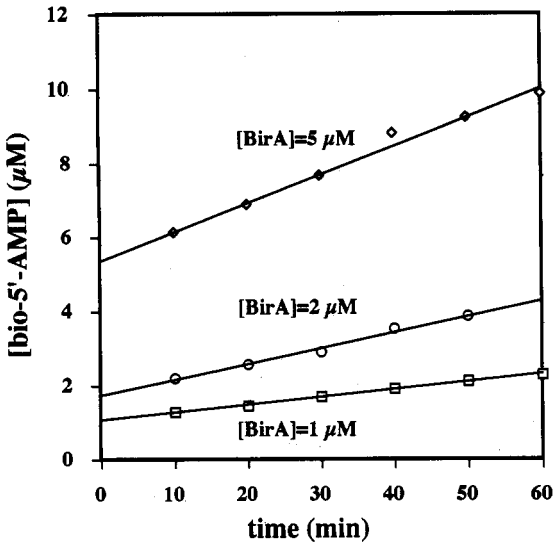

FIGURE 3: Dependence of the slow phase of BirA-catalyzed synthesis of bio-5′-AMP on BirA concentration. Bio-5′-AMP synthesis was quantitated as described in the text. [ATP] = 501 μM and [biotin] = 106 μM. All reactions contained inorganic pyrophosphatase at a final concentration of 2 units/mL and were carried out in buffer A at 20 °C.

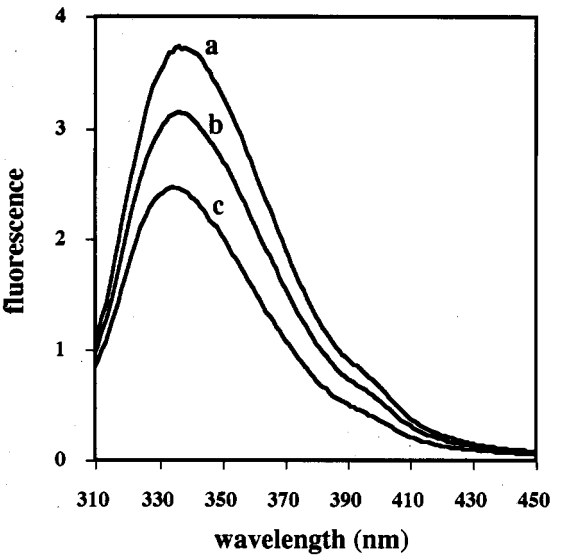

FIGURE 4: Steady-state fluorescence emission spectra of BirA in (a) the absence of ligands and in the presence of (b) biotin and (c) bio-5′-AMP. [BirA] = 0.9 μM in all spectra, [biotin] = 50 μM, and [bio-5′-AMP] = 3 μM. All spectra were obtained in buffer A at 20 °C.

Table 1: Stoichiometry and Rate Constants (k_2) for Dissociation of the BirA–Bio-5′-AMP Complex

BirA (μM)	ATP (μM)	biotin (μM)	n^a	k_2 ($s^{-1} \times 10^{-4}$)^a
5	501	106	1.0 ± 0.1	2.9 ± 0.5
2	501	106	1.0 ± 0.1	4.3 ± 0.5
1	501	106	1.0 ± 0.1	3.4 ± 0.5

^{a} The values represent the average of six ([BirA] = 5 μM), four ([BirA] = 2 μM), and four ([BirA] = 1 μM) measurements.

is unacceptably low, while at concentrations higher than 5 μM, significant amounts of BirA dimer form. We have found that the dimerization of BirA and the binding of bio-5′-AMP are thermodynamically coupled processes (Y. Xu and D. Beckett, unpublished results) and that the apparent value of k_2 decreases with an increasing fraction of dimeric BirA in the species population. The equilibrium dimerization constant of the BirA–bio-5′-AMP complex is approximately 1×10^{-5} M. Both biotin and ATP concentrations utilized in these experiments were sufficiently high to be outside of the concentration-dependent range for measurement of k_2. Measurements of the linear phase of the time course of bio-5′-AMP synthesis over a range of BirA concentrations are shown in Figure 3. The slopes of the lines are, as predicted from the model, proportional to the enzyme concentration. The values of n and k_2 obtained from the data are shown in Table 1. Within experimental error, all values of n are 1. This result agrees with the stoichiometry of the complex determined directly from fluorescence titrations (Nenortas *et al.*, submitted for publication). Only the data obtained at 1 and 2 μM BirA were used to estimate an average value of k_2, because at 5 μM BirA approximately 25% of the total protein is dimeric (Eisenstein and D. Beckett, unpublished results). The lower value of k_2 observed at this concentration reflects the significant contribution of the dimeric species to the species population. The dissociation rate constants determined from the low concentration data are, within experimental error, identical, and the average value of the determinations shown is 0.000 39 ± 0.000 07 s^{-1}. The half-life for the complex calculated from this rate constant is approximately 30 min.

Initial Rate of Bio-5′-AMP Synthesis. The initial rate of bio-5′-AMP synthesis was monitored by stopped-flow fluorescence. Steady-state fluorescence emission spectra of BirA and BirA bound to biotin and bio-5′-AMP are shown in Figure 4. The binding of biotin and bio-5′-AMP results in 15% and 40% quenching, respectively, of the intrinsic BirA fluorescence signal. This difference in the spectra of the unliganded and liganded forms of BirA can be exploited to monitor the initial rate of BirA-catalyzed synthesis of bio-5′-AMP. The equilibrium dissociation constant for the BirA–biotin interaction previously has been shown to be in the 10 nM concentration range (Nenortas *et al.*, submitted for publication). Initial rates were measured at a saturating biotin concentration (50 μM) by rapid mixing of the BirA–biotin complex with ATP and monitoring the resulting change in fluorescence with time. The high concentrations of ATP present in the reactions necessitated the use of an excitation wavelength of 300 nm.

A time course of the initial burst of bio-5′-AMP synthesis is shown in Figure 5. Nonlinear least-squares analysis of the data indicates that the process is best described by a single-exponential model. We are confident that the observed fluorescence change reflects the synthesis of bio-5′-AMP and not the binding of ATP, since the total amplitude of the fluorescence change associated is consistent with that observed in equilibrium titrations of BirA with bio-5′-AMP. All measurements of the initial rate were performed at ATP concentrations sufficiently high to satisfy the condition that the substrate concentration be in great excess of the enzyme concentration. These ATP concentrations are still, however, within the linear range of the rate versus ATP concentration profile. At any given ATP concentration, at a saturating biotin concentration, analysis of the time course of bio-5′-AMP synthesis yields an apparent rate constant, k_{app}, for the process, where k_{cat} is the first-order rate constant for bio-5′-AMP

$$k_{app} = \frac{k_{cat}[ATP]}{[ATP] + K_M} \quad (4)$$

synthesis, and K_M is the Michaelis constant for ATP. If we assume that [ATP] $\ll K_M$, measurement of k_{app} over a range of ATP concentrations permits the determination of the rate constant, k_{cat}/K_M, for BirA-catalyzed synthesis of bio-5′-AMP at saturating biotin concentration (Figure 6). The results of

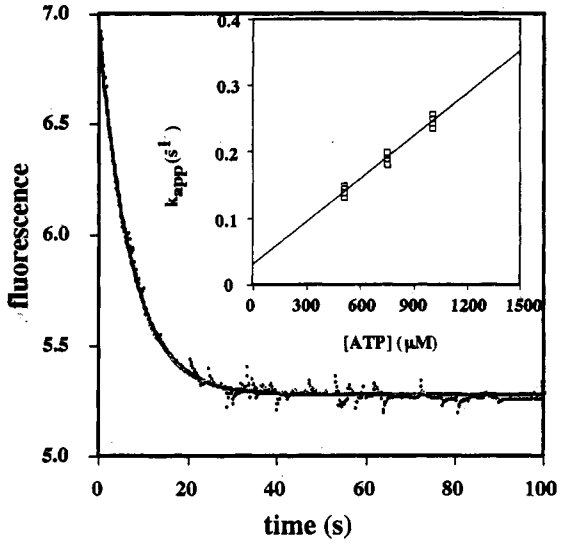

FIGURE 5: Stopped-flow measurement of the intrinsic BirA fluorescence intensity over the time course of the initial burst of BirA-catalyzed synthesis of bio-5′-AMP at saturating biotin concentration. The reaction was carried out in buffer A at 20 °C. [BirA] = 2 μM, [biotin] = 50 μM, and [ATP] = 509 μM. The total amplitude of the fluorescence change represents 23% of the initial BirA–biotin fluorescence signal and is consistent with the results of steady-state fluorescence measurements shown in Figure 4. Inset: Dependence of the apparent rate constant (k_{app}) for BirA-catalyzed synthesis of bio-5′-AMP on [ATP]. [BirA] = 2 μM, [biotin] = 50 μM, and [ATP] = 509, 748, or 1003 μM. Analysis of the data by linear least-squares yields the constant, k_{cat}/K_M.

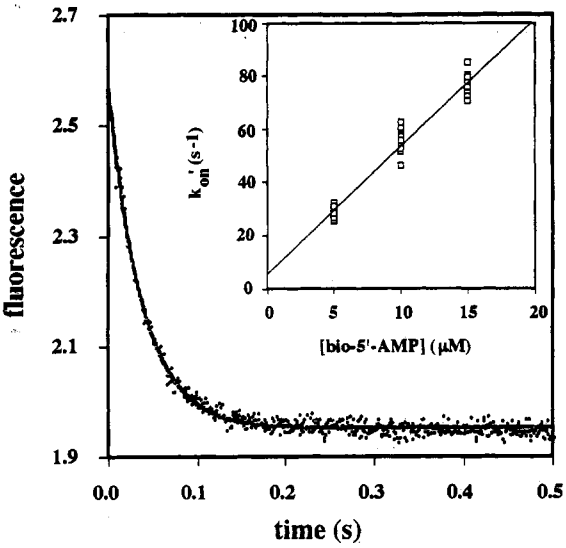

FIGURE 6: Stopped-flow measurement of the bimolecular association of BirA with bio-5′-AMP. The reaction was carried out in buffer A at 20 °C. [BirA] = 1 μM and [bio-5′-AMP] = 5 μM. The solid line represents the curve simulated using the best-fit parameters for the process obtained from nonlinear least-squares analysis of the data using a single-exponential model. Inset: Dependence of the apparent bimolecular rate constant (k_{on}') for association of bio-5′-AMP with BirA on bio-5′-AMP concentration. [BirA] = 1 μM and [bio-5′-AMP] = 5, 10, or 15 μM. Linear least-squares analysis of the data yields the rate constant, k_{on}, for the bimolecular rate process.

this analysis indicate an apparent rate constant of 2.1×10^2 M^{-1} s^{-1}. The fact that the rate versus concentration profile has not reached a plateau with respect to [ATP] indicates that the K_M for ATP is high. Indeed, Lineweaver–Burk analysis of the ATP concentration dependence of the initial rate of bio-5′-AMP synthesis indicates that the K_M for ATP is approximately 3 mM. On the basis of this value of K_M and the value for k_{cat}/K_M of 2.1×10^2 M^{-1} s^{-1}, k_{cat} is calculated to be 0.6 s^{-1}. This apparent rate constant is approximately 2000-fold greater than the value determined for the k_2 above, and the difference provides additional justification for application of the linear form of the kinetic model in eq 3.

Thermodynamic Stability of the BirA–Bio-5′-AMP Complex. Previous attempts to estimate the equilibrium dissociation constant (K_D) for the BirA–bio-5′-AMP interaction using direct equilibrium binding techniques provided only information about the stoichiometry of the complex (Nenortas *et al.*, submitted for publication). Although the results indicated an upper limit for the K_D in the nanomolar concentration range, no accurate determination of the binding constant could be made. The results of kinetic measurements presented above, however, enable determination of the dissociation rate constant of the BirA–bio-5′-AMP complex. Direct measurement of the bimolecular rate constant for formation of the complex, coupled with the dissociation rate information, permits calculation of the equilibrium constant for the interaction. The rate of association of BirA with bio-5′-AMP was measured using stopped-flow fluorescence. BirA and chemically synthesized bio-5′-AMP were mixed rapidly, and the decrease in intrinsic protein fluorescence intensity was monitored with time. A stopped-flow trace resulting from one such measurement is shown in Figure 6. The results of nonlinear least-squares analysis of the data indicate that the process is well-described by a single-exponential model (Figure 6). The total fluorescence change associated with the process, determined from the initial fluorescence intensity of the protein alone and the final fitted value of the fluorescence obtained from analysis of the data, is approximately 40% and is consistent with that determined in equilibrium fluorescence titrations of BirA with bio-5′-AMP. The results of measurement of the apparent bimolecular association rate constant as a function bio-5′-AMP concentration are shown graphically in Figure 6. The association rate constant for the interaction, k_{on}, estimated from these data is $(4.9 \pm 0.7) \times 10^6$ M^{-1} s^{-1}. This value is low relative to the values measured for other bimolecular association processes involving proteins and small ligands (Hammes & Schimmel, 1970).

The dissociation rate constant obtained from the measurement of the time course of bio-5′-AMP synthesis discussed above and the directly measured value of the association rate constant were combined to calculate the equilibrium constant for the protein–ligand interaction using the relationship $K_D = k_{off}/k_{on}$, where K_D is the equilibrium dissociation constant and k_{off} and k_{on} are the unimolecular dissociation and bimolecular association rate constants, respectively. k_{off} corresponds to k_2 in the mechanism of bio-5′-AMP synthesis described above. The results of this calculation yield an apparent equilibrium dissociation constant for the binding of BirA to bio-5′-AMP of $(7.9 \pm 1.7) \times 10^{-11}$ M. The Gibbs free energy calculated from this equilibrium constant of -13.6 ± 0.2 kcal/mol indicates that the complex is very stable.

DISCUSSION

This work represents the first direct measurement of the kinetics of biotinyl-5′-adenylate synthesis catalyzed by a biotin ligase. Although the ligases have previously been the subjects of biochemical studies, detection of the ligase reaction typically has been monitored by quantitation of the transcarboxylation reaction catalyzed by the biotin-accepting protein (Lane *et al.*, 1965; Cazzulo *et al.*, 1970, 1971). We are interested, however, in the thermodynamic and structural features of the BirA–bio-5′-AMP complex and, more specifically, in the regulation of its functional switch from biotin ligase to

transcriptional repressor. Quantitative measurements of the bio-5′-AMP synthesis reaction can provide insight into these features of BirA function. The broad dynamic range of the phosphorimaging system makes it well-suited for the measurement of the bio-5′-AMP synthesis reaction, since low levels of product can be quantitated in the presence of the very high ATP concentrations used in the experiments described in this work.

The time course of bio-5′-AMP synthesis is characterized by a rapid exponential burst followed by a slow linear phase. The burst phase corresponds to the synthesis of 1 mol of the adenylate per mole of enzyme, and the slow linear phase is rate-limited by the slow dissociation of bio-5′-AMP from BirA. The characteristics of this reaction are similar to those observed for the analogous reaction catalyzed by aminoacyl-tRNA synthetases (Fersht, 1975). In that case, the burst phase corresponds to the rapid synthesis of the aminoacyladenylate, while the slow phase reflects the slow hydrolysis of the adenylate. The similarity of the mechanisms found in the tRNA synthetases and BirA may reflect similarities in the chemistry of the overall reactions catalyzed by these two classes of enzymes. Both involve acyl activation via formation of an adenylate, followed by nucleophilic attack of the acyl intermediate by a moiety on the final acceptor molecule. In the case of the tRNA synthetases, the nucleophile corresponds to the 3′-OH on the CCA terminus of the tRNA, while in the case of BirA the ϵ-amino group of a lysine residue on BCCP is the nucleophile.

Rates of the Burst and Linear Phases. We have measured the rate of the burst phase at saturating biotin concentration and have used these results to calculate a rate constant for the synthesis of bio-5′-AMP as approximately 0.6 s^{-1}. This value is low relative to the values of k_{cat} measured for many other enzymes (Fersht, 1985). Conditions of saturating biotin concentration were used because of the difficulties associated with detection of the reaction at lower concentrations. The value for k_2 determined from the linear portion of the time courses is also slow, and the half-life for the BirA–bio-5′-AMP complex calculated from this rate constant is approximately 30 min. At least two reasons exist for the unusually high kinetic stability of the BirA–bio-5′-AMP complex. The first is related to the chemical nature of the acyladenylate, which is a mixed anhydride and may, therefore, undergo hydrolysis in aqueous solution. If the adenylate were readily released into solution, it would be wasted instead of being utilized in either the biotin ligation reaction or repression. Perhaps more important than the issue of waste is the loss of stringent linkage between the two BirA functions that would occur if the adenylate were readily released into solution. The high kinetic stability of the BirA–bio-5′-AMP complex ensures that the most probable state of BirA is the adenylate-bound form. The biotin- or ATP-bound forms are kinetically much less stable. The protein is, therefore, always activated either for transfer of biotin to the BCCP or for binding to the biotin operator sequence. A consequence of always being "on" is that once the intracellular pool of apo-BCCP is converted to holo-BCCP, BirA is in the form required for tight DNA binding. The protein, therefore, automatically shifts from its enzymatic to its transcriptional regulatory function in response to the cellular requirement for biotin.

Thermodynamics of the Interactions of BirA with Biotin and ATP. The kinetic data described in this work, combined with direct measurement of the BirA–biotin interaction, have allowed the estimation of the thermodynamic parameters for interactions of BirA with biotin and ATP. Independent measurement of the equilibrium dissociation constant for the BirA–biotin interaction indicates that it is in the 10–100 nM biotin concentration range (Nenortas *et al.*, submitted for publication). The K_M's for biotin measured for other holotranscarboxylase synthetases range from nanomolar to micromolar in concentration (Lane *et al.*, 1964; Cazzulo *et al.*, 1971; Siegel *et al.*, 1965; Shenoy *et al.*, 1988). If we assume that the K_D for biotin binding is in the same range as the K_M, BirA lies within the middle range relative to other biotin ligases. Measurement of the initial rate of bio-5′-AMP synthesis as a function of ATP concentration indicates that the conditions used for the synthesis are still within the linear range of the rate versus [ATP] profile. This result suggests that the K_M for ATP is in the millimolar concentration range. This high value is much greater than that observed for other holotranscarboxylase synthases (Cazzulo *et al.*, 1971; Siegel *et al.*, 1965; Shenoy *et al.*, 1988), but is consistent with the estimated intracellular ATP concentration of 3 mM (Kornberg & Baker, 1991).

The Bimolecular Rate of Association of BirA with Bio-5′-AMP. The rate constant for the bimolecular association of BirA with bio-5′-AMP is 4.9×10^6 M^{-1} s^{-1}. This value is low relative to both the estimated diffusion-controlled rate and values measured for a large number of protein–small ligand interactions (Hammes & Schimmel, 1970). The slow rate of association may reflect the occurrence of a slow conformational change upon binding of the adenylate to BirA. Two independent lines of evidence support this idea. First, the occurrence of a conformational shift in BirA upon the binding of bio-5′-AMP has been detected using a combination of fluorescence spectroscopy and partial proteolysis techniques (Nenortas *et al.*, submitted for publication). Second, the energetics of BirA with respect to dimerization and sequence-specific DNA binding change upon the binding of bio-5′-AMP. The binding of the adenylate is positively coupled to both of these other binding processes. This change in the energetic state of the protein molecule may be accompanied by a significant change in the structure of BirA.

Thermodynamic Stability of the BirA–Bio-5′-AMP Complex. The measured values of the bimolecular association rate constant for the formation of the BirA–bio-5′-AMP complex and the unimolecular rate constant governing dissociation of the complex have been utilized to estimate the Gibbs free energy for binding of BirA to bio-5′-AMP. The calculated equilibrium dissociation constant for the interaction is 7.9×10^{-11} M, and the Gibbs free energy estimated from this equilibrium constant is −13.5 kcal/mol. The BirA–bio-5′-AMP complex is, thus, quite stable. This high stability of the complex precludes direct measurement of the equilibrium constant by fluorescence titration since, at the lowest protein concentration at which an acceptable intrinsic protein fluorescence signal is obtained, the binding conditions are in the stoichiometric range (Nenortas *et al.*, submitted for publication). The high kinetic stability of the complex also precludes direct measurement of the dissociation rate constant by fluorescence using biotin as a competitor, since the length of time required to perform the measurement is unacceptably long (approximately 5 h). In this time period, considerable photobleaching of the intrinsic protein fluorescence signal was observed (Y. Xu, unpublished observations). Provided that the experiments are performed using the appropriate conditions outlined in the Results section, measurements of the slow linear phase of the time course of bio-5′-AMP synthesis do provide

the dissociation rate information. The concentrations of substrates used in the measurements of k_2 presented in this work are sufficiently high to justify the linear simplification of the time course. The results of measurements of the BirA concentration dependence of the time course agree with the predictions of the model. The model is, moreover, confirmed by results obtained with preloading of the enzyme with a stoichiometric amount of the unlabeled adenylate.

SUMMARY

We have developed a method for measuring the time course of bio-5′-AMP synthesis, which has allowed us to propose a kinetic mechanism for the synthesis of the adenylate. The time course consists of an initial burst phase corresponding to the synthesis of 1 mol of product per mole of enzyme, followed by a slow linear phase that reflects the slow release of bio-5′-AMP from BirA. This slow release of product can be rationalized in terms of the two functions of the BirA–bio-5′-AMP complex: transfer of biotin to the BCCP and binding to the biotin operator sequence. The dissociation rate information obtained from measurements of the time course of bio-5′-AMP synthesis was combined with direct measurements of the bimolecular association rate constant for complex formation to obtain an estimate for the thermodynamic stability of the BirA–bio-5′-AMP complex. The results of this analysis indicate that the complex is extremely stable at both the thermodynamic and the kinetic level. These results suggest that *in vivo* BirA is always activated for either biotin transfer to BCCP or sequence-specific binding to DNA, which provides a mechanism for tight coupling of the two functions of the BirA–bio-5′-AMP complex.

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