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Biochemical Properties and Biological Function of a Monofunctional Microbial Biotin Protein Ligase

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Abstract

Biotin protein ligases constitute a family of enzymes that catalyze biotin linkage to biotin-dependent carboxylases. In bacteria these enzymes are functionally divided into two classes; the monofunctional enzymes that only catalyze biotin addition and the bifunctional enzymes that also bind to DNA to regulate transcription initiation. Biochemical and biophysical studies of the bifunctional *Escherichia coli* ligase suggest that several properties of the enzyme have evolved to support its additional regulatory role. Included among these properties are the order of substrate binding and linkage between oligomeric state and ligand binding.

In order to test this hypothesized relationship between functionality and biochemical properties in ligases, we have carried out studies of the monofunctional ligase from *Pyrococcus horikoshii*. Sedimentation equilibrium measurements to determine the effect of ligand binding on oligomerization indicate that the enzyme exists as a dimer regardless of liganded state. Measurements performed using ITC and fluorescence spectroscopy indicate that, in contrast to the bifunctional *Escherichia coli* enzyme, substrate binding does not occur by an obligatorily ordered mechanism. Finally, thermodynamic signatures of ligand binding to the monofunctional enzyme differ significantly from those measured for the bifunctional enzyme. These results indicate a correlation between the functional complexity of biotin protein ligases and their detailed biochemical characteristics.

Biotin-dependent carboxylases in all organisms utilize biotin to mediate carboxyl group transfer reactions. An example of this class of enzyme is acetyl-CoA carboxylase, which catalyzes the conversion of acetyl-CoA to malonyl-CoA, the first committed step of fatty acid biosynthesis. In its coenzyme function biotin is covalently linked to the biotin carboxyl carrier protein, BCCP, of the carboxylase *via* an amide linkage between the carboxylic acid group of the valerate chain of the coenzyme and the epsilon amino group of a specific lysine side chain of BCCP. The post-translational biotin modification is catalyzed by biotin protein ligases (BPL) in the following two-step reaction:

in which the activated biotin, bio-5'-AMP, is first synthesized by BPL from substrates biotin and ATP and the biotin is subsequently transferred to the lysine residue of the BCCP moiety of the apocarboxylase(1).

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Two classes of BPLs have been identified in microorganisms (2,3). Enzymes in the first class, of which the *Pyrococcus horikoshii* enzyme is a member, only catalyze post-translational biotin addition. The second class includes the well-characterized *Escherichia coli* enzyme, which functions not only as a post-translational modification enzyme but also as a transcriptional repressor (Figure 1(4,5)). As a repressor the *E. coli* enzyme prevents transcription initiation at the two promoters of the biotin biosynthetic operon(4,5). Thus, the *E. coli* enzyme both funnels biotin into metabolism and regulates expression of the genes that code for biotin biosynthetic enzymes. Both classes of BPLs are found throughout the eubacterial and archaeal kingdoms(2,3).

The primary sequences and high-resolution structures of representatives of the two classes of microbial biotin protein ligases indicate similarities as well as striking differences. All microbial biotin holoenzyme ligase sequences are characterized by a homologous region of approximately 250 amino acid residues, which is required for catalytic function. Additionally, the bifunctional ligases possess an N-terminal segment that encodes a winged helix-turn-helix DNA binding domain (2). High-resolution three-dimensional structures of representatives of both classes of ligases have been obtained using x-ray crystallography (6-10). Comparison of the structures of the *E. coli* and *P. horikoshii* enzyme monomers reveals, with the exception of the DNA binding domain at the N-terminus of the *E. coli* enzyme, great similarity (Figure 2). However, the structures diverge at the quaternary level. Structures of the unliganded proteins indicate that while the *E. coli* enzyme is a monomer, the *P. horikoshii* enzyme self-associates *via* extension of the central β -sheet at its N-terminus forming an elongated dimer. Liganded forms of the *P. horikoshii* enzyme show this same dimeric structure. By contrast, the *E. coli* enzyme is dimeric only in structures in which it is bound to biotin or to an analog of the intermediate in the reaction, bio-5'-AMP(6-8). Moreover, the surface utilized for dimerization is completely distinct (Figure 2) from that employed by the *P. horikoshii* enzyme. The *E. coli* holoBirA homodimer is the active species in site-specific DNA binding that is associated with its transcription repression function(11,12).

Biochemical studies of the *E. coli* biotin protein ligase suggest that the detailed mechanistic properties of the enzyme have evolved to support its bifunctionality. First, kinetic measurements of bio-5'-AMP synthesis from biotin and ATP indicate an obligatorily ordered mechanism for substrate binding with biotin binding first followed by ATP(13). This mechanism is supported by structural data that indicate that the ATP binding site is formed as a consequence of biotin-induced folding of specific loop segments in BirA (7). In addition, formation of the adenylate from ATP and biotin is a prerequisite to stable BirA dimerization in solution. Sedimentation equilibrium measurements indicate that while apoBirA and BirA-biotin dimerize very weakly, the adenylate-bound protein dimerizes in the micromolar range of protein concentration. (14) Since BirA homodimerization is required for DNA binding, the thermodynamic coupling of self-association to adenylate synthesis combined with the ordered mechanism of biotin and ATP binding renders DNA binding itself subject to regulation by intracellular biotin concentration (12). Finally, a single surface of the *E. coli* enzyme is used for both homo-dimerization with another holoBirA monomer and hetero-dimerization with apoBCCP (15-17). This feature has consequences for regulating switching between enzymatic and site-specific DNA binding functions.

The pathway of evolution of bifunctionality in biotin holoenzyme ligases is not known. However, it is likely that the coding sequence for the DNA binding domain was linked to that for the catalytic function through a gene fusion event. Biochemical and biophysical studies of the *E. coli* enzyme indicate these two structural elements are functionally coupled through formation of the active adenylate bound enzyme, holoBirA, which both catalyzes biotin transfer and binds sequence specifically to DNA to repress transcription initiation

(18). The obligatorily ordered mechanism of biotin binding followed by ATP renders formation of the activated species exquisitely sensitive to biotin concentration (13). We hypothesize that the monofunctional enzymes, which only transfer biotin to apocarboxylases and play no regulatory role, do not employ this ordered mechanism.

In this work the relationship between mechanistic properties and functionality in microbial biotin protein ligases is investigated in the monofunctional *P. horikoshii* ligase. Measurements of the assembly state of the enzyme in the absence and presence of ligand indicate that, regardless of ligation state, it is dimeric in solution. Measurements of biotin and ATP binding using combined isothermal titration calorimetry and fluorescence spectroscopy indicate that the enzyme can bind each substrate independent of the second and that in binding the individual substrates no interaction between the two sites in the dimer exists. Furthermore, measurements of substrate binding, ATP or biotin, in the presence of saturating concentrations of the second substrate indicate no linkage in binding of the two substrates to the active site in each monomer. The combined results indicate that in mono and bifunctional biotin protein ligases the mechanistic properties of the enzymes correlate with the complexity of their functional properties.

Materials & Methods

Chemicals & Biochemicals

All chemicals used in buffer preparation were at least reagent grade. The d-biotin, adenosine 5'-triphosphate, and α,β -methyleneadenosine 5'-triphosphate were purchased from Sigma Aldrich. Solutions of ATP and the ATP analog were prepared by dissolving the dried powder in H₂O followed by adjusting the pH to 7.5. The resulting solutions were divided into 1 mL aliquots that were stored at -70°C . The nucleotide concentration was determined by UV absorption spectroscopy using an extinction coefficient of $15,900\text{ M}^{-1}\text{cm}^{-1}$ at 259 nm. Biotin solutions were prepared by dissolving the desired amount of dried powder in Standard Buffer (10 mM Tris-HCl, 500 mM KCl, 2.5 mM MgCl₂) that had not yet had its pH adjusted. The pH of the resulting solution was adjusted to 7.5 and the solution was brought to its final volume in a volumetric flask. After passing the solution through a 0.22 μm filter it was divided into 1.0 mL aliquots that were stored at -70°C . The bio-5'-AMP was synthesized and purified as previously described (1,11).

Pyrococcus horikoshii Biotin Protein Ligase Purification

The plasmid encoding the *P. horikoshii* biotin protein ligase, which was obtained from RIKEN, is a derivative of pET-11a in which expression of the *PhBPL* coding sequence is under control of the T7 promoter. The plasmid was transformed into *E. coli* strain BL21- λ DE3 RIL. All cell growth was performed in LB media supplemented with 100 $\mu\text{g/mL}$ ampicillin. Starter cultures were produced by diluting 5 mL of an overnight culture into 50 mL of LB/ampicillin and grown while shaking at 37°C . A one liter volume of media was inoculated with 20 mL of starter culture and incubated at 37°C until the OD₆₀₀ reached a value of 0.9 at which time a fresh solution of IPTG was added to the culture to a final concentration of 75 $\mu\text{g/mL}$ and cell growth was continued for an additional four hours. All manipulations following cell growth, with the exception of a single heating step, were performed at 4°C . The cells were harvested by centrifugation at 4°C at 6000 rpm for 30 minutes, resuspended in 20 mM TrisHCl, 0.5 M NaCl, pH 8.0 at 4°C and lysed by sonication. After removal of cellular debris by centrifugation at 9,000 rpm for 30 minutes the supernatant was treated with polyethylene imine (0.2% vol/vol final concentration) to remove nucleic acids. Following separation of the precipitate by centrifugation at 9,000 rpm for 30 minutes the resulting supernatant was heated at 91°C for 11 minutes to denature contaminating proteins. The precipitated contaminating proteins were pelleted by

centrifugation for 30 minutes at 9,000 rpm and the resulting supernatant was combined with 3 volumes of saturated ammonium sulfate and stirred gently at 4°C overnight. The ammonium sulfate precipitate was separated from the supernatant by centrifugation at 9000 rpm for 30 minutes and the resulting pellet was resuspended in 20 mLs of buffer containing 50 mM TrisHCl pH 7.5 at 4°C, 50 mM KCl, 5% vol/vol glycerol (SP starting buffer). After dialyzing the sample extensively against the same buffer it was loaded onto a 20 mL column packed with SP sepharose resin (Perkin Elmer) at a flow rate of 0.5 mL/minute. The column was washed extensively with the starting buffer at the same flow rate and the protein was eluted in a gradient of 50-500 mM KCl, (Total volume=200 mL). Samples were analyzed by SDS polyacrylamide gel electrophoresis and those containing the pure protein were pooled and dialyzed against buffer containing 50 mM TrisHCl, 200 mM KCl, 5% vol/vol glycerol pH 7.5 at 4°C. The pure protein was stored in 1 mL aliquots in silanized 1.5 mL eppendorf tubes at -70°C. The protein concentration was determined spectrophotometrically using an extinction coefficient at 280 nm of 23470 M⁻¹cm⁻¹(19).

Analytical Ultracentrifugation

The assembly state of the *Ph*BPL was determined using analytical ultracentrifugation in a Beckman-Coulter XL-I analytical ultracentrifuge. All measurements were performed using 6-hole charcoal filled epon centerpieces (1.2 cm) equipped with sapphire windows. The protein was first dialyzed exhaustively against Standard Buffer (10 mM TrisHCl, pH 7.5 at 20°C, 500 mM KCl, 2.5 mM MgCl₂) and then diluted to the appropriate concentration for centrifugation. Samples containing protein alone or protein with saturating concentrations of biotin or bio-5'-AMP were centrifuged at speeds ranging from 20,000 to 24,000 rpm. Scans were acquired at 280 nm (apo-*Ph*BPL and biotin bound) or 295 nm (bio-5'-AMP bound) at a step size of 0.001 nm with 4 averages per step. The data scans were analyzed with WinNonLin(20) using a single species model to obtain a reduced molecular weight, σ , from which a molecular weight was calculated using the following equation:

$$\sigma = \frac{M(1 - \bar{v}\rho)\omega^2}{RT}$$

in which M is the molecular weight in g/mole, $\bar{v}$ is the partial specific volume of the protein calculated from the amino acid composition (SedenTerp, <http://www.rasmb.bbri.org>), ρ is the solvent density determined pycnometrically, ω is the angular velocity of the rotor and R is the gas constant.

Isothermal Titration Calorimetry

Isothermal titration calorimetry was performed using a MicroCal VP-ITC instrument (GE Healthcare). The protein was prepared for measurements by exhaustive dialysis against the appropriate buffer and then diluted into the dialysis buffer to the final desired concentration, degassed, and placed in the sample cell. Ligand was prepared by dilution of the stock into the same buffer used for the dialysis and placed in the syringe. Titrations were performed by injecting the concentrated ligand solution into the protein solution and stirring at a rate of 310 rpm. The protein concentration in the sample cell ranged from 2-4 μ M and the ligand concentration in the syringe was 20-40 μ M. Binding data were analyzed using a simple 1:1 binding model using Origin (GE Healthcare).

Fluorescence Spectroscopy

All fluorescence titrations were performed using an ISS PC1 spectrofluorometer equipped with a circulating water bath to maintain constant temperature. The excitation wavelength

was 295 nm and emission spectra were acquired from 310-450 nm. The excitation and emission bandpasses were set at 8nm and 8nm, respectively. Spectra were acquired as the average of 10 iterations with a step size of 1 nm and scan rate of 1 nm/s. Both the total intensity (corrected for buffer contribution) and the first moment of each spectrum were recorded. Titrations were performed by pipetting small aliquots of ligand into the protein in a quartz cuvette, stirring manually and allowing the system to reach equilibrium by incubating for 2 minutes prior to acquiring spectra. Simple titrations of the *PhBPL* with ATP, the ATP analog and biotin were performed as well as titrations with biotin in the presence of saturating ATP analog and ATP analog in the presence of saturating biotin. Data from titrations were analyzed by nonlinear least squares analysis in Prism 4 (GraphPad) using a simple binding model to obtain the equilibrium association constant for binding and the fluorescence signal (total intensity or spectrum moment) for the ligand free and bound protein. The temperature dependencies of ATP and biotin binding were measured by performing titrations in buffer prepared at the indicated temperatures.

Results

PhBPL is always a dimer in solution

Structures of several forms of the *P.horikoshii* biotin protein ligase have been solved by x-ray crystallography. These include the apo or unliganded enzyme, the enzyme bound to ADP, biotin and bio-5'-AMP (9). In all of these structures the protein is a dimer. However, the dimeric state in the crystal structure may not extend to the protein in solution at low total concentration. The solution state assembly properties of *PhBPL* were measured using sedimentation equilibrium. Protein samples prepared at multiple loading concentrations were centrifuged at multiple speeds and the data were globally analyzed using a single species model. Results of analysis of data obtained for the unliganded protein, which has a monomer molecular weight of 26 kD, indicate that is well modeled as a single species with a molecular weight of 51.2 ± 0.2 kD (Figure 3). Measurements performed in the presence of saturating biotin or bio-5'-AMP also yielded molecular weights consistent with a dimer. Thus, regardless of ligation state, the *PhBPL* is a dimer in solution.

Biotin binding by PhBPL

Biotin binding to *PhBPL* was monitored using steady state fluorescence emission spectroscopy. The intensity of intrinsic protein fluorescence signal of the ligase, the sequence of which contains three tryptophan residues per monomer, is quenched by 30% percent upon addition of saturating concentrations of the substrate biotin (Figure 4A). Furthermore, the emission maximum shifts to the blue by approximately 5 nm. These spectral changes were exploited to monitor biotin binding. Titration of the protein with the ligand yields an isotherm that is consistent with a simple binding process (Figure 4A). Nonlinear least squares analysis of the data using a simple binding model yields an equilibrium constant of $2.0(\pm 0.1) \times 10^{-7}$ M at 20°C (Table 1).

The organism *Pyrococcus horikoshii* was isolated from deep sea thermal vent and grows optimally at 98°C(21). Although it is technically impossible to measure biotin binding at this optimal growth temperature, titrations could be performed over a range of temperatures up to 50°C. Results of these measurements indicate that the binding adheres to a simple mechanism over the entire temperature range and that the equilibrium dissociation constant increases by approximately four fold on increasing the temperature from 10°C to 50°C (Table 1). Analysis of the dependence of the equilibrium association constant on temperature using the van't Hoff formalism yields an enthalpy of binding of -4.4 ± 0.2 kcal/mole. Direct titrations of the enzyme with biotin performed using isothermal titration calorimetry at 20°C confirm this binding enthalpy and yield an equilibrium association for

the binding process identical to that measured by fluorescence titrations (Figure 5). Finally, measurement of the temperature-dependence of binding by ITC reveals a heat capacity change of 5 ± 14 cal/mole·K, consistent with the assumption of the van't Hoff analysis that the binding enthalpy is temperature-independent (Figure 7B).

ATP binding

Saturation of the protein with ATP results in quenching of 60% of the fluorescence signal of the protein and a blue shift of approximately 5 nm (Figure 6A). These fluorescence changes enabled titrations to determine the affinity and binding mode for ATP. A titration performed at 20°C yields data that are well-described by a simple binding model and nonlinear least squares analysis revealed an equilibrium dissociation constant of $2.4(\pm 0.3) \times 10^{-4}$ M. Titrations with ATP performed over a temperature range from 20 to 50°C revealed a modest decrease in the affinity as the temperature increases (Table 2) and van't Hoff analysis of the dependence of the equilibrium constant on temperature yields a binding enthalpy of -3.5 ± 1.5 kcal/mole (Figure 6B).

Linkage between biotin and ATP binding

Binding of ATP and biotin to *PhBPL* leads to enzyme-catalyzed synthesis of the intermediate, bio-5'-AMP. In the *E. coli* enzyme no ATP binding occurs in the absence of biotin binding. By contrast, as indicated from the titrations described above, ATP does bind to the *PhBPL* in the absence of biotin. However, it is not known if each substrate affects the binding of the second substrate to the enzyme. Thermodynamic coupling in ATP and biotin binding to *PhBPL* was investigated by performing binding titrations with each ligand in the presence and absence of the second ligand. Fluorescence spectroscopy was used to monitor the progress of the titrations.

In order to avoid the chemistry that occurs upon binding of the biotin and ATP substrates an ATP analog was used in the linkage measurements. The analog in which a methylene replaces the oxygen that links the α and β phosphates in ATP, α,β -methylene adenosine 5'-triphosphate, was used. The effect of this structural modification on binding of the nucleotide was first investigated by measuring binding of the analog alone to the enzyme. As observed with ATP, addition of saturating amounts of the analog to *PhBPL* results in quenching of the intrinsic protein fluorescence. However, in contrast to ATP binding, which results in a blue shift in the maximum of the fluorescence spectrum, binding of the analog resulted in a red shift. Titration of *PhBPL* with the analog yields binding data that are well-described by a simple 1:1 model with a resolved equilibrium dissociation constant of $1.5(\pm 0.2) \times 10^{-4}$ M at 20°C, a value similar to that measured for ATP binding in the same buffer conditions (Table 3).

The linkage between ATP and biotin binding was investigated by measuring binding of each ligand in the presence of saturating amounts of the second ligand. Titration of the *PhBPL*-biotin complex with the ATP analog yields data that when subjected to nonlinear least squares analysis using a simple binding model, reveals an equilibrium constant identical to that obtained from measurements of ATP and the ATP analog alone (Table 3). Likewise, titration of the protein presaturated with the ATP analog with biotin also yields an equilibrium dissociation constant for biotin binding identical to that obtained under the same conditions for biotin alone (Table 3). Thus, there is no thermodynamic linkage in biotin and ATP binding to the *P. horikoshii* enzyme.

Discussion

Dimeric structure of PhBPL is independent of ligation state

In all *PhBPL* structures determined by x-ray crystallography the protein is dimeric (9). In this work equilibrium analytical ultracentrifugation measurements performed on the apo-enzyme and its complexes with biotin and bio-5'-AMP all yield molecular weights for the protein consistent with a dimer. Thus, regardless of ligation state the *P. horikoshii* enzyme is a dimer. Furthermore, the lack of evidence of any monomer in the sedimentation equilibrium measurements is consistent with a very stable dimer interface.

The oligomerization properties of monofunctional biotin protein ligases are not conserved. High resolution-structures of other monofunctional biotin protein ligases are available including those from *Mycobacterium tuberculosis*, and *Aquifex aeolicus* (10). Although at the monomer level these two proteins are structurally similar to the *P. horikoshii* enzyme, the oligomeric state can either be monomeric or dimeric. Moreover, in the dimeric enzymes observed in the crystal structures the surface utilized for dimerization is not conserved. The *P. horikoshii* enzyme dimerizes using the N-terminal β -strand of the large-sheet that characterizes the core of enzyme active site domain (Figure 2). By contrast, in the *M. tb* dimer the C-terminal SH3-like domain is utilized in dimerization (2CGH). Moreover, size exclusion chromatography measurements indicate that this dimer is not stable (22).

Homodimer formation by the bifunctional *E. coli* biotin protein ligase is coupled to bio-5'-AMP binding (14). Kinetic measurements of bio-5'-AMP synthesis by the *E. coli* monomer indicate that substrate binding in adenylate synthesis occurs *via* an obligatorily ordered process in which biotin binding precedes ATP binding(13). Consequently, the activation of dimerization by bio-5'-AMP binding renders dimerization and, therefore, transcription regulation sensitive to biotin concentration (Figure 1A). In addition, the surface used for BirA homodimerization is identical to that used for heterodimerization with apoBCCP (Figure 2). This mutually exclusive nature of the homo- and hetero-dimerization provides a mechanism for regulation of the functional switch between post-translational modification enzyme and transcriptional repressor(16,17). By contrast, the oligomerization properties of the monofunctional ligases do not appear to serve any regulatory function.

Thermodynamic parameters governing Biotin and ATP binding to PhBPL

The thermodynamics of biotin binding to *PhBPL* indicate relatively modest affinity. The equilibrium dissociation constant governing biotin binding at 20°C is approximately 200 nM. Additionally, the relatively modest temperature dependence of binding suggests that even at the optimal growth temperature for *P. horikoshii* of 98°C the equilibrium dissociation constant will be approximately 1.6×10^{-6} M. In *E. coli* the total biotin concentration is estimated to be in the nanomolar range of the concentration. Assuming similar intracellular biotin concentration in *P. horikoshii* and that the K_m for biotin binding is equal to the K_D , the substrate concentration is considerably lower than the K_M . In addition to the Gibbs free energy of biotin binding, the enthalpy of the interaction is modest at -4.4 kcal/mole. The result of this modest enthalpy is that even at the optimal growth temperature of 98°C the protein still has considerable affinity for the substrate. Biotin binding to the *E. coli* enzyme is characterized by an enthalpy of approximately -20 kcal/mole at 20°C (Figure 7a) (23). Nonetheless, at 37°C the equilibrium dissociation constant governing biotin binding to the *E. coli* ligase is 1.3×10^{-7} M, a value that is 10-fold lower than that predicted for the *P. horikoshii* enzyme at its optimal growth temperature. Finally, while biotin binding to *PhBPL* is characterized by a heat capacity change that is, within error, zero, biotin binding to the *E. coli* enzyme is characterized by substantial heat capacity change of approximately -0.2-0.3 kcal/mole-K (24, 25).

The thermodynamics of biotin binding to two other monofunctional biotin ligases have been determined. Consistent with results for the *PhBPL* described in this work, biotin binding the *A. aeolicus* and the *M. tuberculosis* enzymes is weaker than for the *E. coli* enzyme (10,22). The difference between the monofunctional and bifunctional enzymes potentially facilitates the role of the bifunctional enzyme in regulating biotin biosynthesis, with the greater sensitivity of the *E. coli* enzyme to biotin concentration enabling feedback of increased biotin concentration to transcription repression at the biotin biosynthetic operon.

In contrast to the bifunctional *E. coli* enzyme, to which ATP binding cannot be detected in the absence of biotin binding, the nucleotide substrate binds to the *P. horikoshii* enzyme on its own. Measurements of the affinity indicate a K_D in the 200-400 micromolar range of concentration in the temperature range of 20-50°C. Moreover, the binding enthalpy is modest. Consequently, the affinity at the optimal growth temperature of 98°C is anticipated to be similar to the values reported in this work. The independence of ATP and biotin binding to the *PhBPL* is consistent with observations made on the monofunctional *Aquifex aeolicus* BPL. Both of these enzymes share several structural features with the *E. coli* enzyme including a biotin binding loop and an adenylate binding loop. In the *E. coli* enzyme this latter loop is disordered in both the apo-enzyme structure and the biotin-bound structure and is ordered only in the complex of the protein bound to the adenylate analog, biotinol-5'-AMP. By contrast, the loop is ordered in the structures of the unliganded forms of both monofunctional enzymes(9,10). It has been proposed that this ordering renders the nucleotide binding site "pre-organized" in the monofunctional enzyme thereby allowing nucleotide binding independent of biotin.

No coupling is observed in ATP and biotin binding to the *PhBPL*. The affinities for biotin and the ATP analog, α,β -methyleneadenosine 5'-triphosphate are identical regardless of the occupancy of the site by the second ligand. This result is somewhat surprising in the context of the structural data available for the enzyme. In the absence of any ligand the biotin binding loop of the enzyme is disordered. Structures of the biotin and ADP-bound protein indicate ordering of the loop. One might anticipate that the pre-ordering that is the consequence of binding of one substrate would facilitate binding of the second substrate.

Previously published results combined with the results described in this work suggest that both coupling between biotin and ATP binding and the order of substrate binding are correlated with ligase functionality. The bifunctional *E. coli* enzyme does not bind ATP in the absence of biotin(13 and recent direct binding studies performed by KGD). By contrast, both the *P. horikoshii* and *A. aeolicus* enzymes bind nucleotide in the absence of biotin. In addition, the micromolar equilibrium dissociation constants for ATP binding by the monofunctional ligases are significantly lower than the K_m for ATP in *E. coli* BPL-catalyzed bio-5'-AMP synthesis of approximately 3 mM (13). Linkage in binding of the two substrates has been measured for the *A. aeolicus* enzyme. However, these measurements are inconclusive because while they indicate no coupling when ATP binding is measured in the presence of saturating biotin, a coupling free energy of approximately -1.0 kcal/mole was estimated from measurements of biotin binding in the presence and absence of ATP (10). The discrepancy may result from the fact that, in order to avoid chemistry, binding measurements were performed in the absence of Mg^{2+} , an essential cofactor in catalysis of bio-5'-AMP synthesis that is included in all buffers used in the measurements reported in this work (1). These authors concluded that, consistent with the *P. horikoshii* enzyme, substrate addition to the *A. aeolicus* enzyme occurs *via* a random mechanism. By contrast, the *E. coli* enzyme functions in an obligatorily ordered mechanism, which renders biotin concentration a trigger for accumulation of the activated BirA:bio-5'-AMP species (Figure 1).

Monofunctional and bifunctional microbial biotin ligases have evolved distinct mechanisms of substrate binding and self-association. The mono-functional ligases are either constitutive monomers or dimers. Moreover, in these enzymes dimerization is independent of substrate or intermediate binding. By contrast, the bifunctional *E. coli* ligase is a conditional homo-dimer that depends on formation of the adenylylated intermediate and is utilized solely for the transcriptional regulatory function. Substrate binding mechanisms are also distinct for the two classes of BPLs with the monofunctional ligases exhibiting ATP binding in the absence of biotin and a random mechanism of ATP and biotin binding. This contrasts with BirA, which requires biotin binding prior to nucleotide binding. Superficially, the two classes of ligases are similar in both primary sequence and tertiary structure. Previous studies suggest that the distinct dimerization properties of the bifunctional enzyme results from evolution of the sequences of surface loops on the protein (26,27). The distinct substrate binding properties of the monofunctional and bifunctional enzymes may also be related to properties of the adenylylate binding loop. While the loop in BirA is disordered in the absence of ligand, in the *P. horikoshii* and *A. aeolicus* enzymes it is ordered. This ordered loop conformation may confer on the monofunctional ligases both the ability to bind nucleotide in the absence of biotin and the resulting random mechanism of substrate binding.

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Abbreviations

PhBPL	<i>Pyrococcus horikoshii</i> Biotin Protein Ligase
E. coli	<i>Escherichia coli</i>
ITC	Isothermal titration calorimetry
CoA	coenzymeA
BCCP	Biotin carboxyl carrier protein
ATP	adenosine 5'-triphosphate
Tris	Tris hydroxyl methyl amino methane
IPTG	Isopropyl- β -D-thiogalactoside

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FIGURE 1.

(A) The monofunctional *PhBPL* biotinylates the biotin carboxyl carrier protein (BCCP) subunit of biotin-dependent carboxylases. (B) The bifunctional *BirA* can biotinylate BCCP or form a homodimer that acts as a transcriptional repressor of biotin biosynthetic genes.

FIGURE 2.

(A) *P. horikoshii* BPL monomer. (B) BirA monomer. (C) *P. horikoshii* BPL exists as a constitutive dimer. (D) The BirA homodimer. Models were constructed in MolMol (28) with Protein Databank Files 1WPY (A and C) and 2EWN (B and D). The *Ph*BPL monomer in (A) and the BirA monomer in (B) were each obtained by deletion of the second monomer from their respective dimer models.

FIGURE 3.

Sedimentation equilibrium concentration distribution for *PhBPL*. Concentration profiles were obtained from samples prepared at 5.3, 10.7, and 18 μM protein monomer in Standard Buffer and centrifuged at 20,000($\circ$) and 24,000($\square$) rpm. The solid lines represent results of global non-linear least-squares analysis of six data sets. The lower panel indicates the residuals of the fit.

FIGURE 4.

Biotin binding to *PhBPL*. (A) Normalized binding data obtained by titrating *PhBPL* with biotin at 20°C. The solid line represents the best-fit obtained from non-linear least-squares analysis of the data using a single-site binding model. (A) inset, fluorescence emission spectra acquired for *PhBPL* (solid line) and *PhBPL*-biotin (dotted line). (B) Van't Hoff analysis of the temperature dependence of the equilibrium association constant for the *PhBPL*-biotin interaction was obtained from titrations performed from 10 to 50°C. The solid line represents the best-fit of the data to the van't Hoff equation.

FIGURE 5.

Calorimetric titration of *PhBPL* with biotin obtained by ITC from thirty-three $9\mu\text{L}$ injections of $40\mu\text{M}$ ligand into a $4\mu\text{M}$ protein solution in Standard Buffer at 20°C . The solid line represents the best-fit of the data to a single-site binding model.

FIGURE 6.

ATP binding to *PhBPL*. (A) Normalized binding data obtained from titration of *PhBPL* with ATP at 20°C. The solid line represents the best-fit obtained from non-linear least-squares analysis of the data using a single-site binding model. (A) inset, fluorescence emission spectra acquired for *PhBPL* (solid line) and *PhBPL*·ATP (dotted line). (B) Van't Hoff analysis of the temperature dependence of the equilibrium association constant for the *PhBPL*-ATP interaction was obtained from titrations performed from 20 to 50°C. The solid line represents the best-fit of the data to the van't Hoff equation.

FIGURE 7.

Thermodynamics of biotin binding to *PhBPL* and *BirA* are distinct. (A) The values of ΔG° (checkered) and ΔH° (black) for biotin binding to *PhBPL* and *BirA*(24) at 20°C were obtained from isothermal titration calorimetry. The $-T\Delta S^\circ$ (white) values were calculated using the equation $\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$. (B) Temperature dependence of biotin binding obtained either in this work from full titrations of *PhBPL* (λ) or in previous work using the total association at partial saturation (TAPS) method for *BirA*(τ)(24). The solid lines represent the best-fit of the data to the equation $\Delta C_p^\circ = d\Delta H/dT$.

TABLE 1 Temperature dependence of biotin binding to *PhBPL*

Temperature (°C) ^a	K _D (M) ^b	ΔG° (kcal/mol) ^c
10	1.2(±0.3)×10 ⁻⁷	-8.96±0.01
20	2.0(±0.1)×10 ⁻⁷	-8.90±0.04
30	2.0(±0.2)×10 ⁻⁷	-9.20±0.05
40	5.1(±0.8)×10 ⁻⁷	-8.93±0.09
50	3.9(±0.7)×10 ⁻⁷	-9.4±0.1

^a Measurements were performed in Standard Buffer (500 mM KCl, 10mM TrisHCl, 2.5mM MgCl₂, pH 7.5±0.2 at the indicated temperature) as described in Materials & Methods.

^b Errors correspond to the standard deviation of results obtained from at least two independent experiments.

^c The free energy, ΔG°, is related to the equilibrium constant, K_D, by the expression $\Delta G^\circ = RT \ln K_D$.

TABLE 2 Temperature dependence of ATP binding to *PhBPL*

Temperature (°C) ^a	K _D (M) ^b	ΔG° (kcal/mol) ^c
20	2.4(±0.3)×10 ⁻⁴	-4.80±0.08
30	2.9(±0.9)×10 ⁻⁴	-4.86±0.02
40	3.0(±0.2)×10 ⁻⁴	-5.00±0.04
50	4.3(±0.9)×10 ⁻⁴	-4.9±0.1

^aMeasurements were performed in Standard Buffer (500 mM KCl, 10mM TrisHCl, 2.5mM MgCl₂, pH 7.5±0.2 the indicated temperature) as described in Materials & Methods.

^bErrors correspond to the standard deviation of results obtained from at least two independent experiments.

^cThe free energy, ΔG°, is related to the equilibrium constant, K_D, by the expression $\Delta G^\circ = RT \ln K_D$.

TABLE 3

Linkage between biotin and ATP binding to *PhBPL*

Ligand ^a	K _D (M) ^d	ΔG° (kcal/mol) ^e
ATP	2.4(±0.3)×10 ⁻⁴	-4.80±0.08
AMPCPP	1.5(±0.2)×10 ⁻⁴	-5.09±0.08
AMPCPP/Biotin ^b	1.4(±0.3)×10 ⁻⁴	-5.1±0.1
Biotin	2.0(±0.1)×10 ⁻⁷	-8.90±0.04
Biotin/AMPCPP ^c	2.6(±0.3)×10 ⁻⁷	-8.74±0.07

^aMeasurements were performed in Standard Buffer (500 mM KCl, 10mM TrisHCl, 2.5mM MgCl₂, pH 7.5±0.2 at the 20°C temperature) as described in Materials & Methods.

^bα,β-methyleneadenosine 5'-triphosphate was titrated into *PhBPL* saturated with biotin.

^cBiotin was titrated into *PhBPL* saturated with α,β-methyleneadenosine 5'-triphosphate.

^dErrors correspond to the standard deviation of results obtained from at least two independent experiments.

^eThe free energy, ΔG°, is related to the equilibrium constant, K_D, by the expression ΔG° = RTlnK_D.