The C-terminal domain of biotin protein ligase from *E. coli* is required for catalytic activity

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Abstract

Biotin protein ligase of *Escherichia coli*, the BirA protein, catalyses the covalent attachment of the biotin prosthetic group to a specific lysine of the biotin carboxyl carrier protein (BCCP) subunit of acetyl-CoA carboxylase. BirA also functions to repress the biotin biosynthetic operon and synthesizes its own corepressor, biotinyl-5'-AMP, the catalytic intermediate in the biotinylation reaction. We have previously identified two charge substitution mutants in BCCP, E119K, and E147K that are poorly biotinylated by BirA. Here we used site-directed mutagenesis to investigate residues in BirA that may interact with E119 or E147 in BCCP. None of the complementary charge substitution mutations at selected residues in BirA restored activity to wild-type levels when assayed with our BCCP mutant substrates. However, a BirA variant, in which K277 of the C-terminal domain was substituted with Glu, had significantly higher activity with E119K BCCP than did wild-type BirA. No function has been identified previously for the BirA C-terminal domain, which is distinct from the central domain thought to contain the ATP binding site and is known to contain the biotin binding site. Kinetic analysis of several purified mutant enzymes indicated that a single amino acid substitution within the C-terminal domain (R317E) and located some distance from the presumptive ATP binding site resulted in a 25-fold decrease in the affinity for ATP. Our data indicate that the C-terminal domain of BirA is essential for the catalytic activity of the enzyme and contributes to the interaction with ATP and the protein substrate, the BCCP biotin domain.

Keywords: Biotin protein ligase; biotin holoenzyme synthetase; ATP binding; protein-protein interactions; posttranslational modification

Biotin protein ligase (EC 6.3.4.15), also known as holocarboxylase synthetase, catalyses the covalent attachment of the biotin prosthetic group to a specific lysine of the biotin carboxyl carrier domain of biotin-dependent carboxylases in a two-step reaction:

This reaction is conserved throughout biology, with the enzymes from different species able to recognize and correctly biotinylate carboxylases from widely divergent sources (Cronan and Wallace 1995; Leon-Del-Rio et al. 1995; Tissot et al. 1996), albeit with differing affinities (Polyak et al. 2001). The biotin carboxylases catalyze key reactions in essential metabolic processes and, thus, not only these enzymes but also the biotin ligases, are essential for survival (Chapman-Smith and Cronan 1999a).

The biotin protein ligase of *Escherichia coli* is the 35.3 kDa BirA protein. BirA is able to recognize and quantita-

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Abbreviations: BCCP, biotin carboxyl carrier protein; IPTG, isopropyl-1-thio- β -D-galactopyranoside; PAGE, polyacrylamide gel electrophoresis; S.D., standard deviation.

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tively biotinylate only one of the > 4000 protein species in *E. coli*, the biotin carboxyl carrier protein (BCCP) subunit of acetyl-CoA carboxylase. Despite the detailed structural, biochemical, and genetic analyses of the BirA protein, little is known about how the ligase recognizes its protein substrate and specifically modifies a unique lysine residue. Unlike its eukaryotic counterparts, BirA also functions to repress the biotin biosynthetic operon and is unusual among DNA-binding proteins in that it synthesizes its own corepressor, biotinyl-5'-AMP, the catalytic intermediate in the biotinylation reaction (Eisenberg et al. 1982; Chapman-Smith and Cronan 1999b).

The three-dimensional structure of *E. coli* BirA has been solved by X-ray crystallography in the absence of substrates and with biotinyl-lysine bound at the active site (Wilson et al. 1992). The N-terminal 60 residues of the protein form a helix-turn-helix DNA-binding domain. The central domain has considerable sequence homology with the eukaryotic biotin protein ligases (Tissot et al. 1997; Chapman-Smith and Cronan 1999a) and contains the residues that contact biotin in the crystal structure (Wilson et al. 1992) and, thus, is believed to form the catalytic site. Several loops within this domain are disordered in the crystal structure, and the conformational changes that accompany formation of the BirA:biotinyl-5'-AMP complex (Xu et al. 1995; Xu and Beckett 1996; Beckett and Matthews 1997) probably include alterations in these structurally undefined regions of the protein. One loop, comprising residues 211–223, contains a subtilisin-sensitive site C-terminal to S217 that is protected against cleavage in the presence of biotinyl-5'-AMP (Xu et al. 1995). This observation implies that the structural transition that follows the first catalytic step includes a conformational change within this loop, or that the presence of the reaction intermediate impedes access of subtilisin to the cleavage site. The disordered loop containing residues 115–123 lies close to the biotin binding site, and residues 115–118 become ordered in the crystal structure when biotinyl-lysine occupies the active site. Because of the similarity to nucleotide-binding sequences in protein kinases, the sequence ¹¹⁵GRGRRG¹²⁰ within this loop had been thought to function in ATP binding (Buoncristiani et al. 1986; Wilson et al. 1992). However, a recent mutational analysis shows that this sequence has a role in biotin binding (Kwon and Beckett 2000). No function has been identified previously for the C-terminal domain comprising residues 274–321, which shows structural similarity to the Src homology 3 domains (Noble et al. 1993). However, recent evidence of protection by biotinyl-5'-AMP against hydroxyl radical cleavage of the BirA backbone at several sites within the C-terminal domain implies a possible role in the enzyme reaction (Streaker and Beckett 1999). Unfortunately, there is no high-resolution structural information yet available for the form of BirA that is recognized by the BCCP biotin domain, i.e., the BirA:biotinyl-5'-AMP complex.

The structure of the apo and holo forms of the biotin domain of BCCP has been solved by X-ray crystallography and NMR spectroscopy, giving essentially identical structures (Athappily and Hendrickson 1995; Roberts et al. 1999). The domain is a barrel structure consisting of two antiparallel β -sheets each containing four strands, with the N- and C-termini close together at one end and the biotinyl-lysine exposed on a tight β -turn on the opposite face of the molecule. Full-length BCCP has an additional N-terminal region of 70 to 80 residues, presumed to be the dimerization and intersubunit interaction domain for assembly of the functional acetyl-CoA carboxylase (Li and Cronan 1992).

We have previously isolated and characterized two charge substitution mutants in the biotin domain of BCCP, E119K, and E147K (Chapman-Smith et al. 1999). E147K BCCP is poorly biotinylated by BirA, whereas the E119K substitution virtually abolishes biotinylation. Our analysis showed these to be genuine ligase interaction mutants rather than structurally defective proteins and suggested that matching charged surfaces may be important in recognition of the BCCP biotin domain by BirA. Other observations support the importance of charge in the interaction. Replacing a conserved PMP motif in the biotin domain of human propionyl-CoA carboxylase with PKP has a more pronounced effect on biotinylation than replacing all three residues with alanine (Leon-Del-Rio and Gravel 1994), and replacing either of the Met residues flanking the biotinyl lysine with Lys greatly reduces biotinylation of BCCP in *E. coli* (Reche et al. 1998). Furthermore, one of the few derived constraints in a biotinylation consensus sequence selected from a random peptide library is for either Glu or Asp at the position equivalent to E119 in BCCP (Schatz 1993).

In the present study, we inspected the molecular surfaces of the available structures (Wilson et al. 1992; Roberts et al. 1999) to identify basic residues in BirA that could potentially interact with E119 and E147 of BCCP. Our mutational analysis of residues selected in this way indicated that the C-terminal domain of BirA is essential for the catalytic activity of the enzyme and contributes to the interaction with both ATP and the protein substrate BCCP.

Results

Expression and purification of BirA

Initial experiments with expression of BirA from our pET-based vector pHBA (see Materials and Methods) produced highly variable levels of protein. We also observed inconsistent revival from cryostorage of BL21(λ DE3) strains harboring pHBA. It seemed likely that this was a consequence of toxicity resulting from overexpressing a DNA-binding protein under the control of the notoriously leaky T7lac promoter. Therefore, glucose was included in the media before induction and cultures were grown at reduced tem-

perature (see Materials and Methods) to minimize synthesis of BirA during the preinduction phases. This simple approach allowed consistent expression of BirA (Fig. 1, lanes 1,2). Applying the cleared cell lysate directly to a Fast-Flow resin under conditions in which BirA binds allowed rapid removal of nucleic acid and most cellular proteins as a first step in the purification (Fig. 1, lanes 3,4). Ion exchange chromatography using elution with KCl gradients maintained high levels of enzyme activity during purification. Overall, the rapid two-step procedure gave essentially pure material (Fig. 1, lane 5) with specific activities of 350–400 mU/mg protein. The enzyme is stable at 4°C for extended periods and retains activity for at least several years if stored at –80°C.

Mutagenesis of BirA

Design: We performed manual docking of the molecular structures of BirA (Wilson et al. 1992) and the biotin domain of BCCP (Roberts et al. 1999) to identify by inspection Lys and Arg residues in BirA that may interact with E119 and E147 in BCCP (Fig. 2). Because of the nature of the structural information available for BirA, this process had inherent limitations. First, the known structures of BirA were of the free and biotinyl lysine-bound forms, rather than the form that is recognized by the BCCP biotin domain, i.e., the BirA:biotinyl-5'-AMP complex, and significant conformational changes are known to accompany biotin binding and adenylate formation (Xu et al. 1995; Xu and Beckett 1996; Beckett and Matthews 1997). Second, the crystal structure of apoBirA contains several disordered loops in

Fig. 1. Expression and purification of BirA analyzed by SDS-PAGE. Samples were separated on a 10% polyacrylamide gel under reducing conditions using the Tris-Tricine buffer system (see Materials and Methods). M: molecular mass markers (Mark 12, Novex). Lane 1: crude extract before induction. Lane 2: crude extract after induction with IPTG. Lane 3: material passing through the S-Sepharose column. Lane 4: peak of enzyme activity eluting from S-Sepharose. Lane 5: peak of enzyme activity eluting from Q-Sepharose.

Fig. 2. 3D structure of the BCCP biotin domain showing the positions of the biotinylated lysine, K122, and residues E119 and E147. The figure was generated using MOLSCRIPT, version 2.1.1 (Kraulis 1991), and Raster3D, version 2.4 (Merritt and Bacon 1997), with Protein Data Bank file 2BDO as input.

the central catalytic domain adjacent to the biotin binding site (Wilson et al. 1992; Fig. 3). More recently, determination of the structure of the biotin-bound form of BirA shows that three of these loops become ordered following biotin binding as predicted from the biochemical data (Weaver et al. 2001). Given the BirA structures available at the time, we considered that the extensive regions of disorder around the biotin binding pocket precluded rigorous model building. Furthermore, only a single point of contact between BirA and BCCP at the biotin moiety within the active site could be defined accurately. Thus, the computer-assisted selection of potential interacting residues involved rotating BCCP about this single fixed point within the BirA structure using InsightII software (Molecular Simulation). Despite these limitations, this approach identified a relatively small number of candidate basic residues across a broad face of the BirA molecule where contact with E119 or E147 of BCCP would be possible. The initial purpose of this study, and the focus when designing the mutations, was to define the interactions between BCCP and BirA rather than investigating other effects on catalytic activity. Therefore, candidate residues that contact biotin in the crystal structure (Wilson et al. 1992), those in the KWPND biotin binding motif also found in the biotin-binding protein avidin (Tissot et al. 1997), and those within and immediately adjacent to the presumptive ATP binding motif, ¹¹⁵GRGRRG¹²⁰, were avoided. At the time our mutants were designed, it was thought that the ¹¹⁵GRGRRG¹²⁰ sequence was involved in ATP binding, although the study published during the course of our work identifies this sequence as binding biotin (Kwon and Beckett 2000).

Eight positively charged residues in BirA identified in this way (K122, R182, R212, R213, K277, K283, R317, and K321) were substituted with glutamate by site-directed mutagenesis (see Materials and Methods). Because the modeling suggested that the C-terminal domain of BirA could potentially play a role in recognition of the BCCP biotin domain, a mutant truncated at I272, between the central catalytic domain and the C-terminal domain (Wilson et al.

Fig. 3. Views of the 3D structure of *E. coli* BirA highlighting features relevant to this study. The *left* panel shows the N-terminal domain (purple), the central domain (blue) with biotin bound at the active site, and the C-terminal domain (green). The position of residues substituted by site-directed mutagenesis that are located within the structured regions of the protein, R182, I272, K277, K283, and R317, is indicated. Additional mutated residues that lie within unstructured regions are K122, R212, R213, and K321. The *right* panel shows biotin bound in the active site cleft of the enzyme and highlights the position of R317 and K277 relative to the biotin binding site. The ends of the loops comprising residues 118–125 and 211–223 are indicated by numbering on both panels. The *left* panel was generated using MOLSCRIPT, version 2.1.1 (Kraulis 1991), and Raster3D, version 2.4 (Merritt and Bacon 1997), and the *right* panel using InsightII (MSI) with Protein Data Bank file 1BIB as input.

1992), was constructed. In addition, we constructed G115S BirA, the *birA1* variant originally isolated by Barker and Campbell (1981) and investigated further by Kwon and Beckett (2000). The positions of the mutated residues within the BirA structure are shown in Figure 3.

Analysis: Crude cell lysates prepared from cultures expressing the mutant BirA proteins were analyzed for enzymatic activity with wild-type, E119K, and E147K forms of the BCCP biotin domain (see Materials and Methods). Control experiments using lysates derived from cells harboring empty vector showed no detectable activity from endogenous BirA under the conditions used. None of the complementary charge substitution mutations in BirA restored activity to wild-type levels when assayed with the mutant substrates (Fig. 4). This suggests that whereas charge is clearly important in the recognition process, a simple interaction between single amino acid residues is not a sufficient determinant. However, several of the introduced mutations altered the catalytic activity of the enzyme with wild-type BCCP as substrate. Both the G115S and R182E substitutions reduced the extent of biotinylation two to threefold with wild-type BCCP in assays using crude material, and the C-terminal truncation mutation, I272X, produced an insoluble protein that lacked activity (Fig. 4). Surprisingly, the R317E mutation within the C-terminal domain had a

strong effect on catalytic activity with wild-type BCCP, reducing the activity ~10-fold.

Although there were no strongly compensatory effects seen with the complementary charge substitution mutations, K277E BirA had higher than wild-type activity with the E119K substrate (Fig. 4), although the absolute activities were low. Furthermore, small effects were observed in some experiments with E147K BCCP for the mutant proteins containing substitutions within the C-terminal domain. As the comparison with wild-type BirA activity in these assays depended on quantitation of the levels of expressed proteins in the crude lysates as bands on SDS-PAGE, the inaccuracy inherent in this approach prevented reliable detection of small changes in affinity of the mutant enzymes for the BCCP biotin domains. In addition, the unexpected effect of the R317E substitution on BirA activity with wild-type BCCP invited further analysis. Taken together, these observations were the first indication that mutations within the C-terminal domain can alter catalytic activity. Previous mutational screens have failed to isolate mutations in this region of BirA (Barker and Campbell 1981; Buoncrisiani et al. 1986; Weaver et al. 2001). Therefore, to assess the effect of the mutations within the C-terminal domain, we purified the BirA proteins containing the K277E, K283E, R317E, or K321E substitutions and performed kinetic analysis in vitro.

Fig. 4. Cleared cell lysates were prepared from *E. coli* strains expressing the wild-type (wt) or mutant forms of BirA as indicated, and enzymatic activity with wild-type (black), E147K (gray), or E119K (white, lower panel) BCCP biotin domains was determined (see Materials and Methods). Note the expanded ordinate scale in the lower panel. I272X denotes a stop codon replacing the native isoleucine at position 272. The amount of BirA protein added to the assays was quantitated by separation of lysates on SDS-PAGE (see Materials and Methods) and is expressed in arbitrary units. Error bars represent the S.D. of three replicate experiments.

The C-terminal domain

The mutant BirA variants were purified as described in Materials and Methods. As a result of the charge substitution, the mutant proteins eluted from both ion exchange columns at different salt concentrations to the wild-type protein (data not shown) and, therefore, could be separated from the endogenous enzyme. Unlike the other variants, the final yield of the K277E mutant was significantly lower than that of the wild type, and the protein showed instability during purification, which is consistent with this substitution having destabilized the structure.

Kinetic constants for the interaction with biotin and MgATP, the substrates in the first step of the biotinylation reaction, were determined for wild-type and the four mutant proteins (see Materials and Methods). The R317E mutation resulted in a 25-fold decrease in affinity for MgATP relative to the wild-type enzyme (Fig. 5; Table 1). A smaller effect was seen for the K277E mutation, with a 6.8-fold increase in K_M for MgATP and no change in k_{cat} . Both of these mutations had a slight effect on the K_M for biotin (Table 1), which was not statistically significant ($p = 0.25$, K277E; $p = 0.20$, R317E). The K283E and K321E substitutions did not affect the kinetics observed when either biotin or MgATP was varied.

Fig. 5. Steady-state analysis of MgATP binding to wild-type and R317E BirA. The activity of the purified wild-type (■) and R317E (△) enzymes was measured at varying levels of MgATP, and kinetic constants were determined from the curves as described in Materials and Methods. The graphs represent one dataset from Table 1. Note the differing ordinate scales.

The activity of the four purified mutant enzymes with wild-type, E119K, and E147K forms of the BCCP biotin domain was compared with wild-type BirA in *in vitro* biotinylation assays at saturating levels of biotin and MgATP (see Materials and Methods). None of the mutations in BirA had a marked effect on the affinity for the wild-type protein substrate (Table 2). Because E119K BCCP is an extremely poor substrate for BirA ($K_M > 200 \mu M$; Chapman-Smith et al. 1999), we were unable to reach saturating levels of this substrate in our assay system and, thus, could not calculate reliable kinetic constants. Instead, the activity of each mutant and the wild-type enzyme was determined at E119K BCCP concentrations below the K_M , and the velocity expressed relative to the enzyme concentration (Fig. 6). This indicated that the K277E mutation in BirA increased the affinity of the enzyme for the E119K mutant substrate by a factor of ~8, whereas the other mutations had no effect. None of the mutations in BirA altered the relative affinity of the enzyme for E147K BCCP compared with wild-type BCCP (Table 2). However, the R317E enzyme had a slightly lower K_M for the wild-type and mutant forms of the biotin domain, suggesting that this mutation slightly enhanced interaction with the protein substrate.

The ATP binding site

Our kinetic experiments suggested that R317 has a role in ATP binding, because the R317E mutation significantly de-

Table 1. Kinetic constants for the interaction of the substrates biotin and MgATP with wild-type and mutant BirA

BirA	MgATP			biotin		
	K_M (mM)	k_{cat} (sec ⁻¹)	$\frac{k_{cat}}{K_M}$ (sec ⁻¹ M ⁻¹) $\times 10^3$	K_M (μ M)	k_{cat} (sec ⁻¹)	$\frac{k_{cat}}{K_M}$ (sec ⁻¹ M ⁻¹) $\times 10^6$
Wild type	0.20 $\pm$ 0.03	0.17 $\pm$ 0.06	0.8 $\pm$ 0.2	0.3 $\pm$ 0.1	0.3 $\pm$ 0.1	1.05 $\pm$ 0.02
K277E	1.37 $\pm$ 0.07	0.2 $\pm$ 0.1	0.16 $\pm$ 0.09	0.6 $\pm$ 0.2	0.3 $\pm$ 0.1	0.4 $\pm$ 0.1
K283E	0.29 $\pm$ 0.07	0.28 $\pm$ 0.06	1.0 $\pm$ 0.2	0.20 $\pm$ 0.02	0.17 $\pm$ 0.04	0.9 $\pm$ 0.3
R317E	5.1 $\pm$ 0.6	0.081 $\pm$ 0.006	0.016 $\pm$ 0.001	0.7 $\pm$ 0.1	0.037 $\pm$ 0.004	0.06 $\pm$ 0.02
K321E	0.28 $\pm$ 0.01	0.32 $\pm$ 0.04	1.1 $\pm$ 0.2	0.27 $\pm$ 0.03	0.28 $\pm$ 0.03	1.03 $\pm$ 0.05

The activity of the purified enzymes using the wild-type BCCP biotin domain as a biotin acceptor was measured under steady state conditions and constants calculated from the data as described in Materials and Methods. The values given are the mean and S.D. of two to four determinations.

creased the affinity of the enzyme for ATP. R317, located within the C-terminal domain, lies some distance from both the biotin binding site and the residues flanking the loop-containing residue S217 (Fig. 3). Previous work has shown that reduced sensitivity to subtilisin at S217 accompanies biotinyl-5'-AMP formation (Xu et al. 1995). The binding of biotin and ATP to BirA is ordered, with biotin binding first, and the presence of biotin confers only a slight degree of protection against subtilisin at S217 compared with biotinyl-5'-AMP (Xu et al. 1995), indicating that restructuring of loop 211–223 requires ATP. This observation is consistent with the recently determined structure of BirA with bound biotin, in which the 211–223 loop remains disordered (Weaver et al. 2001). Therefore, we used limited proteolysis to investigate further the role of R317 and loop 211–223 in ATP binding.

Wild-type and R317E BirA were exposed to subtilisin in the absence and presence of saturating concentrations of biotin and varying concentrations of MgATP, representing the K_M and saturating levels for the two BirA variants. Limited proteolysis, with and without substrates, gave two products detected by SDS-PAGE (Fig. 7A) and identified by mass spectrometry as M1-S217 (mass calculated equals 23612.4, mass determined equals 23614) and V218-K322 (mass calculated equals 11717.6, mass determined equals 11718), indicating cleavage at S217. As expected, preincu-

bation with biotin and MgATP to allow biotinyl-5'-AMP formation protected wild-type BirA and R317E BirA against proteolysis (Fig. 7B). However, the rate of cleavage at S217 at all concentrations of MgATP tested was the same for both the R317E mutant and wild-type BirA. This suggests that the change in the K_M for ATP observed for the R317E mutant reflects a different event in the catalytic pathway from that which results in protection from proteolytic cleavage at S217.

Discussion

This work provides the first information about residues in BirA that interact with the protein substrate and shows that reversal of surface charge on the seemingly distant C-terminal domain of BirA modulates events at the active site. Our data indicate that K277 influences the interaction between BCCP and BirA, which results in biotinylation of the biotin domain (Fig. 6). The observation that the charge substitution in BirA failed to restore activity with E119K BCCP and did not appreciably decrease activity with wild-type BCCP indicates that a direct interaction between E119 of BCCP and K277 of BirA is unlikely. A recent study of hydroxyl radical cleavage of BirA shows that cleavage of the protein backbone from residues K273 to V276 is specifically reduced in the BirA:biotinyl-5'-AMP complex

Table 2. Kinetic constants for the interaction of wild-type and E147K BCCP biotin domains with wild-type and mutant BirA

BirA	apo wild type			apo E147K		
	K_M (μ M)	k_{cat} (sec ⁻¹)	$\frac{k_{cat}}{K_M}$ (sec ⁻¹ M ⁻¹) $\times 10^3$	K_M (μ M)	k_{cat} (sec ⁻¹)	$\frac{k_{cat}}{K_M}$ (sec ⁻¹ M ⁻¹) $\times 10^3$
Wild type	7.0 $\pm$ 0.2	0.44 $\pm$ 0.02	63.1 $\pm$ 0.2	11.2 $\pm$ 0.9	0.24 $\pm$ 0.02	21.6 $\pm$ 0.04
K227E	3.5 $\pm$ 0.1	0.26 $\pm$ 0.09	70 $\pm$ 27	9.0 $\pm$ 0.6	0.25 $\pm$ 0.09	30 $\pm$ 12
K283E	8 $\pm$ 1	0.9 $\pm$ 0.2	115 $\pm$ 7	15 $\pm$ 5	0.8 $\pm$ 0.1	50 $\pm$ 20
R317E	2.5 $\pm$ 0.3	0.12 $\pm$ 0.02	46 $\pm$ 3	3.3 $\pm$ 0.3	0.09 $\pm$ 0.002	29 $\pm$ 4
K321E	6 $\pm$ 2	0.8 $\pm$ 0.2	150 $\pm$ 80	13 $\pm$ 5	0.6 $\pm$ 0.3	50 $\pm$ 20

The activity of the purified enzymes was measured under steady state conditions at saturating concentrations of biotin and MgATP and constants calculated from the data as described in Materials and Methods. The values given are the mean and S.D. of two to four determinations.

Fig. 6. The activity of wild-type and mutant forms of BirA with the mutant E119K BCCP biotin domain. The activity of the purified wild-type (■), K277E (▲), K283E (○), R317E (△), and K321E (●) enzymes was measured at varying levels of E119K mutant BCCP as described in Materials and Methods.

(Streaker and Beckett 1999), indicating that the conformational change associated with the transition between the BirA, BirA:biotin, and BirA:biotinyl-5'-AMP complexes affects the region adjacent to K277. The increased biotinylation of E119K BCCP by K277E BirA may reflect a local conformational change that enhances the interaction of the two mutant proteins. Alternatively, K277 may be involved in the correct alignment of BCCP within the active site of the enzyme. Our observations are consistent with conclusions of Reche et al. (2000) that recognition of BCCP by BirA is topographic and determined predominantly by the tertiary fold. These workers measured changes in chemical shift for residues in BCCP in the presence of BirA by NMR spectroscopy and found that E119K forms part of the surface region of BCCP that shows significant changes in chemical shift in the presence of BirA.

Our assays using unpurified mutant enzymes also detected a small increase in biotinylation by G115S BirA in assays with the E119K BCCP biotin domain substrate (Fig. 4). A kinetic study using purified G115S BirA has shown that this mutation confers an increased K_M for biotin, resulting from an increased rate of dissociation of the BirA:biotinyl-5'-AMP complex (Kwon and Beckett 2000). Thus, it is possible that the observed increase in biotinylation of E119K BCCP in these assays may reflect a non-specific chemical acylation of the protein in the immediate environment of the active site, as has been observed in acyl-AMP transfer by *E. coli* enterobactin synthetase (Ehmann et al. 2000), rather than the usual geometrically specific biotinylation event.

Our data implicate residue R317 of the C-terminal domain in the interaction with the small ligand, ATP. This is consistent with the observation that several specific sites in the C-terminal domain of BirA are protected against hydroxyl radical cleavage in the presence of the reaction intermediate, biotinyl-5'-AMP (Streaker and Beckett 1999). These workers suggest that the observed protection may be a consequence of long-range transmission of allosteric effects, or that the C-terminal domain may play a role in biotin and ATP binding. The present work supports the latter possibility. A recent study of human biotin protein ligase also supports an essential role for the C-terminal domain in enzyme function. Truncations that remove either the entire C-terminal domain, defined by homology with BirA, or the 31 C-terminal residues result in enzyme that is inactive with the bacterial and human biotin domain substrates (Campeau and Gravel 2001). Alignment of the C-terminal domains of *E. coli* BirA with other protobacterial and eukaryotic biotin protein ligases showed that a basic residue is completely

Fig. 7. Proteolytic digestion of wild-type and R317E BirA and protection by MgATP. (A) Digestion of wild-type or R317E BirA in the absence of substrates. Samples were exposed to subtilisin as described in Materials and Methods and digestion products analyzed by SDS-PAGE on 12% polyacrylamide gels using the Tris-Tricine buffer system. The time (min) after addition of subtilisin is shown above each lane. The sizes of the digestion products indicated to the right were determined by mass spectrometry (see Materials and Methods). M: molecular mass markers (Mark 12, Novex). (B) Protection by MgATP. Wild-type and R317E BirA were incubated without (squares) or with 100- μ M biotin and MgATP at 0.3 mM (triangles), 5 mM (circles), or 20 mM (diamonds) before addition of subtilisin. The amount of intact BirA remaining at each time was quantitated after separation of the products of proteolytic cleavage on SDS-PAGE (see Materials and Methods). Wild-type and R317E BirA are shown by filled and open symbols, respectively.

Fig. 8. Alignment of the C-terminal domain of biotin protein ligases from *E. coli*, *Vibrio cholerae*, *Pasteurella multocida*, *Haemophilus influenzae*, *P. aeruginosa*, *Aribidopsis thaliana*, *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, and *Homo sapiens*. The triangle indicates the start of the C-terminal domain as determined from the 3D structure (Wilson et al. 1992). Conserved amino acid residues are colored white with black background, and similar residues are shaded. R317 of BirA is indicated by the star. Alignment using CLUSTALW; figure produced using Boxshade.

conserved at the position equivalent to R317 of BirA (Fig. 8). In the protobacterial enzymes, the residue at this position is exclusively Arg, whereas in the eukaryotic enzymes, the corresponding residue is exclusively Lys. In addition, in seven of the nine sequences shown, a residue adjacent to the conserved basic residue is also basic, which is consistent with our data suggesting that the positively charged character of this region of the biotin protein ligases has a role in the interaction with ATP. Our alignment is different to that presented by Tissot et al. (1997), which does not show conservation of the basic residues in question. However, we believe that the alignment in Figure 8 better characterizes the similarity between the protobacterial and eukaryotic enzymes. The DG diad from the highly conserved PDGNSFD motif in the eukaryotic enzymes, discussed by Campeau and Gravel (2001), is aligned with the sequence DG in the *Pseudomonas aeruginosa* and *E. coli* enzymes, and additional small residues, acidic residues and hydrophobic residues are aligned across the two subfamilies without the need for the introduction of internal gaps.

The relatively small change in k_{cat} conferred by the R317E substitution indicates that R317 does not function directly in catalysis. R317 may be one of several residues involved in ATP binding or orientation of the substrate or catalytic complex, as seen with arginine residues in other enzymes that use phosphate-containing substrates (Dwyer et al. 2001; Morse et al. 2000). Such a role for R317 is consistent with the magnitude of the K_{M} defect for R317E. For example, a similar substitution at R94 of glutaryl-CoA dehydrogenase, a residue that contributes to binding the substrate glutaryl-CoA, gives rise to a 10- to 16-fold increase in K_{M} (Dwyer et al. 2001). The slightly increased K_{M} for ATP we observed for the K277E mutation may be because of a less-specific effect of an alteration in charge near R317 or may be a consequence of the structural instability resulting from this substitution.

In the complex of BirA with biotin bound, the loop 211–223 remains disordered (Weaver et al. 2001), and protection against subtilisin cleavage at S217 accompanies formation of the BirA:biotinyl-5'-AMP complex (Xu et al. 1995).

Thus, the reduction in subtilisin sensitivity in the presence of biotin and MgATP seen in our experiments required that BirA bind both biotin and ATP and catalyze formation of biotinyl-5'-AMP (Fig. 7). The R317E substitution did not detectably alter the conformation of BirA as assayed by subtilisin cleavage, either in the absence of substrates or in the biotinyl-5'-AMP-bound form, because the rates of cleavage in both cases were the same as wild type (Fig. 7b). Once the R317E BirA:biotinyl-5'-AMP complex had formed in the preincubation period in our assay, the presence of the R317E mutation did not affect the stability of the complex at any of the ATP concentrations tested. The overall reaction catalyzed by BirA involves the formation of several intermolecular complexes accompanied by a series of conformational changes (Xu et al. 1995; Xu and Beckett 1996; Beckett and Matthews 1997; Weaver et al. 2001), and there were several additional reaction steps contributing to the determined kinetic constants in the assay system we used (Fig. 5; Table 1). Our data, showing that subtilisin resistance is unaffected by ATP concentration in R317E compared with wild-type BirA, indicate that R317 exerts its primary effect on catalytic activity at a step other than the stabilization of the BirA:biotinyl-5'-AMP complex. Thus, the observed decrease in affinity for ATP in R317E BirA may be a consequence of a defect in ATP binding and/or biotinyl-5'-AMP formation. It is possible that a charged surface in the region around residues R317 and K277 is involved in a conformational change that stabilizes an interdomain contact needed to form the ATP binding site. Distinguishing between these alternatives may have to await the availability of detailed structural information for these regions of the protein that would be provided by a high-resolution structure of the BirA:biotinyl-5'-AMP complex.

Several systems for overexpressing BirA in *E. coli* have been reported that give varying amounts of purified protein (Buoncrisiani and Otsuka 1988; Xu and Beckett 1997; Reche et al. 1998; O'Callaghan et al. 1999). Producing the protein as a GST-fusion gives the highest yields, up to 50 mg/L culture (O'Callaghan et al. 1999) compared with ~1–10 mg/L culture when producing the isolated protein from

the *tac* promoter (Buoncristiani and Otsuka 1988; Xu and Beckett 1997). O'Callaghan et al. (1999) suggest that this may be due in part to a reduction in the ability of the GST-BirA fusion to bind DNA compared with BirA alone, thus reducing the toxicity associated with overproduction of a DNA-binding protein. Whereas production in a GST-fusion system has advantages for the less quantitative applications of avidin-biotin technologies, we chose not to produce BirA with a fusion partner to avoid potential problems associated with protease treatment when the released protein is required for kinetic studies. This is particularly relevant when producing mutant variants, because these may have altered susceptibility to protease (Chapman-Smith et al. 1999). Instead, the strategy of additional repression of the *lac* promoter, by the presence of glucose and growth at lower temperatures, reduced the deleterious effects on expression attributed to toxicity. In addition, we chose not to use a His-tagged system for purification, because the presence of the tag may have interfered with an analysis of the effect of our charge substitution mutations. Instead, by adapting the published protocols for purification, a rapid, facile method was developed that gave good recovery of pure protein (Fig. 1).

Materials and methods

Expression and purification of wild-type BirA protein

BirA was purified from *E. coli* BL21(ΔDE3) cells transformed with the plasmid pHBA. pHBA was constructed from pBA11 (Barker and Campbell 1981) and pET16b (Novagen) and carries the gene encoding *E. coli* BirA under the control of the T7*lac* promoter. For expression, cells were revived from storage at -80°C onto LB agar supplemented with 2% glucose and 200-μg/mL ampicillin and grown overnight at 30°C . Cells harvested from an overnight culture grown at 30°C in LB, containing 2% glucose and 200-μg/mL ampicillin were resuspended in fresh LB containing 200-μg/mL ampicillin or 100-μg/mL carbenicillin, and 25-mL aliquots used to inoculate 500-mL volumes of the same media. Cultures were grown in shaker flasks at 30°C to $A_{600\text{nm}}$ of 0.5, then the temperature was increased to 37°C and expression induced by addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 0.1 mM. After 2–3 h, cells were harvested, washed, and resuspended in 50-mM sodium phosphate at pH 6.0, 50-mM KCl, 5% glycerol, 0.1-mM dithiothreitol (Buffer A), and lysed with a French pressure cell. Cell-free extract was applied directly to a 50-mL S-Sepharose Fast-Flow column (Pharmacia) equilibrated in Buffer A, and the BirA protein was eluted with a linear gradient of 50 to 500-mM KCl. Fractions containing enzymatic activity eluted at ~300-mM KCl and coincided with the major absorbance peak at 280 nm. This material was pooled and dialyzed overnight against 20-mM Tris-HCl at pH 8.0, 5% glycerol, 0.1-mM dithiothreitol at 4°C and applied to a Q-Sepharose Fast-Flow column (Pharmacia) equilibrated in the same buffer, and the protein was eluted with a linear gradient of 0–400-mM KCl. Fractions containing enzymatic activity eluted at ~120-mM KCl and coincided with the major absorbance peak at 280 nm. This material was dialyzed overnight against 20-mM Tris-HCl at pH

7.5, 200-mM KCl, 5% glycerol, and 0.1-mM dithiothreitol at 4°C and stored at -80°C .

Production of mutant BirA proteins

For mutagenesis, the *birA* coding region was cloned from pHBA into the smaller plasmid, pK18 (Pridmore 1987; Y. Jiang, A. Chapman-Smith, and J.E. Cronan, unpubl.). Site-directed mutagenesis was performed with the QuikChange method (Stratagene), using complementary oligonucleotides encoding the desired mutations synthesized by GeneWorks. For expression of the mutant proteins, the *birA* gene containing each mutant of interest was subcloned back into pHBA, producing variants pHBA1-10. Mutant proteins were expressed as described for wild-type BirA and purified in essentially the same manner. Protein was detected by following the $A_{280\text{nm}}$, and the presence of the enzyme in the peak fractions was confirmed by SDS-PAGE. The mutants eluted from S-Sepharose at 240-mM KCl and from Q-Sepharose at 180-mM KCl.

In vitro biotinylation assays

The apo BCCP biotin domains were purified, and enzyme activity was determined with the biotinylation assay as described previously (Chapman-Smith et al. 1997, 1999), using either cleared cell lysates or purified enzyme. Kinetic constants were determined for each substrate under steady-state conditions at saturating concentrations of the other two substrates, using nonlinear regression analysis as described previously (Chapman-Smith et al. 1999; Polyak et al. 1999, 2001).

Proteolysis

BirA samples, 5 μM, were exposed to subtilisin according to the protocol of Xu et al. (1995) with the following modifications: The final ratio of BirA to subtilisin was 350:1, and the digestion buffer contained 100-mM KCl and included 2.5% glycerol. After addition of subtilisin, duplicate 10-μL samples were removed at the indicated times into 5-μL of 15% acetic acid to terminate the cleavage reaction. The samples were stored at -80°C , and the products of proteolytic digestion detected either by separation on SDS-PAGE or by mass spectrometry.

Protein analysis techniques

Protein methods were essentially as described previously (Chapman-Smith et al. 1997, 1999), except that quantitation after PAGE was performed using NIH ImageQuant software. The concentration of purified BirA was determined using the BioRad Protein Assay kit. Mass spectrometry was performed on a PE Siex API100 electrospray single-quadropole mass spectrometer at the Hanson Centre Protein Core Facility, Adelaide.

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